PHOSPHORUS METABOLISM

IN THE

EMBRYONATED HEN EGG.

A thesis submitted for the Degree

of

DOCTOR OF PHILOSOPHY

by

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APPENDIX II /

INTRODUCTION

The importance of organic phosphates in the metabolism of living organisms has become increasingly obvious as the science of Biochemistry has developed. In the system of the embryonated hen egg the two main sources of phosphorus are the lipids and phosphoprotein of the yolk. From these, the phosphorus of the nucleic acids, the bone salt and so on, in the embryo, must be derived. The object of the work to be described in this thesis was to study how the phosphorus of the yolk phosphoprotein becomes available to the developing embryo.

HISTORICAL:

Although the proteins of egg yolk have been a subject of investigation for at least a hundred years, their first clear classification was not accomplished until the first decade of this century. In 1908 Plimmer and Scott were able, by salt fractionation, to divide the yolk proteins into two markedly different components which they named lipovitellin and livetin. The lipovitellin was shown to contain a quantity of phosphorus which remained after exhaustive extraction with organic solvents and with dilute acid. They investigated the nature of the phosphorus in the solvent-extracted residue from lipovitellin and found that /

that it could be converted completely to inorganic phosphate by treatment with dilute alkali at 37° C for 24 hours, thus resembling the phosphorus of casein, (Plimmer and Bayliss 1906). The non-lipoid phosphorus of lipovitellin (or vitellin phosphorus) was in this quite unlike that of the nucleins, which remained organically bound under this treatment. Vitellin, the lipid free residue from lipovitellin and casein were thereafter classified together as phosphoproteins. The livetin component contained a much smaller proportion of this non-extractable phosphorus and the work of Kay and Marshall (1928) makes it probable that phosphorus found by Plimmer and Scott was due to contaminating lipovitellin.

Plimmer and Scott used the alkaline hydrolysis as the basis of their technique for distinguishing between phosphoprotein phosphorus and nucleic acid phosphorus analytically and so were able to trace the disappearance of phosphoprotein which takes place during the period of development of the embryo in the incubated egg.

After the work of Plimmer and Scott no further studies were made on the metabolism of the yolk phosphoproteins, their chemical structure becoming the focus of interest, especially the nature of the linkage of the phosphorus to the protein. In 1927, Posternak and Posternak found serine among the products of hydrolysis

of /

of vitellin and also large quantities of ammonia and pyruvic acid. They advanced the suggestion that the ammonia and pyruvic acid might come from the degradation of serine residues in the protein molecule and showed that, with this supposition, sufficient serine would be present to account for all the phosphorus of vitellin as linked to the hydroxyl group on the carbon atom of that amino acid. By controlled hydrolysis of vitellin, Lipmann and Levene in 1932 isolated serine phosphate as its barium salt. It was later shown that the rate of hydrolysis of serine phosphate was equal to the rate at which phosphorus was removed as inorganic phosphate from vitellin. The nature of the linkage of phosphate to vitellin was thus established with fair certainty.

The action of proteolytic enzymes on vitellin has been studied by various workers. Posternak and Posternak (1927) and Herd (1937) have been able to isolate from the digests so obtained, polypeptides which contained much higher proportions of phosphorus and of serine than the original vitellin, suggesting either that in the vitellin molecule the phosphoserine residues are grouped close together or that the lipovitellin consists of a mixture or loose complex of a protein or polypeptide rich in phosphoserine residues with a relatively phosphorus free protein. The claim of Mecham and Olcott /

Olcott (1949) to have isolated a protein from egg yolk containing 10% of phosphorus and accounting for 70% of the phosphoprotein phosphorus of the yolk points to the latter suggestion. Young and Phinney (1951), however, have obtained electrophoretically pure lipovitellin containing only 2% of phosphorus and proved it to be the major component of the yolk proteins. The question therefore remains unsettled. It would appear that either Mecham and Olcott have obtained a degraded product or in Young and Phinney's experiments association is occurring.

THE NATURE OF THE PROBLEM ATTEMPTED:

It will be seen from the preceding summary that although the chemical nature of the yolk phosphoprotein has been quite intensively studied and a considerable body of knowledge exists on that aspect, information on its digestion and metabolism in the system of the embryonated hen egg is scanty. The work of Plimmer and Scott (1909) showed that nearly all the phosphorus of the fresh egg was in the form of lipids and phosphoprotein of the yolk and that during the incubation these materials furnished phosphorus for the nucleic acids, ester phosphates and inorganic phosphate of the embryo.

During the course of incubation the yolk is gradually /

gradually enclosed in a highly vascular heavily convoluted structure, the yolk sac membrane. This membrane appears at least superficially to be ideally adapted for absorption from the yolk. The demonstration of a lipase (Ammon and Schütte 1935) and of a proteinase (Borger and Peters 1933; Goldstein and Ginzburg 1937) suggested that the membrane performs digestive, in addition to absorptive, work.

The investigations contemplated when this work was begun in 1949 were:

1) A more precise determination of the rate of utilisation of the phosphoprotein phosphorus and an attempted correlation of this with the growth of the yolk sac membrane.

2) Testing of the yolk itself for the ability to metabolise phosphoprotein.

3) An examination of the role of the yolk sac membrane in phosphoprotein utilisation.

4) A preliminary study of the enzymic equipment available in the yolk sac membrane for the metabolism of organic phosphates.

5) The determination of nucleic acids in the embryo and "Remainder" during the period of embryonic development. This, although not directly connected with the main problem of phosphoprotein metabolism, could be studied, with little extra labour, during the studies /

studies on phosphoprotein level at various stages of incubation.

PART I

THE RATE OF PHOSPHOPROTEIN UTILISATION

INTRODUCTION

The results obtained by Plimmer and Scott (1909) for the phosphoprotein phosphorus remaining at various stages of incubation would appear to give a means of obtaining the rate of phosphoprotein utilisation. The work of Ramsay (1950) on the iron content and Boyd (private communication) on the choline and methionine content of eggs from hens of the same breed on a standard diet has shown, nevertheless, quite wide variations in the amounts of these constituents. It was felt that if a similar variation existed with regard to phosphoprotein, the picture presented by Plimmer and Scott's rather limited number of analyses, although probably qualitatively correct, might be quantitatively misleading. It was therefore decided to carry out an extensive series of analyses for this type of compound at each stage during development. For this, a simple and rapid modification of the method of Schmidt and Thannhauser (1945) was devised.

In the Schmidt and Thannhauser type of analysis, a sample of tissue homogenate or mince is exhaustively extracted with trichloroacetic acid followed by lipid solvents. The residue from this, which should now have the nucleic acids and phosphoprotein as its only phosphorus containing compounds, is after drying incubated /

incubated with normal alkali at 37°C for 16 hours; a process which liberates the phosphorus of phosphoproteins as inorganic orthophosphate, splits ribonucleic acid to nucleotides, but leaves deoxyribonucleic acid precipitable. The amounts of D.N.A.P., R.N.A.P. and phosphoprotein phosphorus can therefore be calculated from the values for total, acid-soluble and inorganic phosphate of the incubate. Two basic modifications in technique were introduced. The first was the use of repeated centrifugation for the extractions, so cutting the number of transferences and effecting an economy in time and apparatus. The second was the adoption of Allen's method (1940) for the actual phosphorus determinations. Based on the method of Fiske and Subbarow (1925) but incorporating the rapid perchloric acid ashing technique of King (1932), Allen's method had already been proved in this laboratory to combine accuracy and rapidity with a desirable degree of robustness. Similar modifications have been made by Davidson and Leslie (1951).

Since the additional work involved in estimating D.N.A.P. and R.N.A.P. by this method was slight, it was decided to extend the scope of the work to include these. It was felt that a comparison of the results obtained with those of Novikoff and Potter (1948), who had used the technique of Schneider (1945), might prove interesting. /

interesting. The work attempted, therefore, was a survey of the nucleic acids and phosphoprotein in the embryo and "remainder" throughout the course of development. The recent work of Davidson, Frazer and Hutchison (1951) showing the difficulty of removing the last traces of acid-soluble phosphorus during the extraction procedure has, however, occasioned doubt as to the accuracy of the small values obtained for phosphoprotein in the embryo and nucleic acid in the "remainder" and these results will consequently not be presented.

EXPERIMENTAL

1. PREPARATION OF MATERIAL:

Eggs weighing 55±5 grammes from the Brown Leghorn flock maintained at the Poultry Research Centre, Edinburgh on a standard breeding diet, were incubated electrically under normal conditions of temperature and humidity. After the desired period of incubation the eggs were candled prior to freezing for ten minutes in a mixture of acetone and solid carbon dioxide. The frozen eggs, stripped of their shells, were rapidly weighed and dissected into embryo and "remainder", the former being weighed and the weight of the latter being obtained by difference. The "remainder" consisted of residual yolk, residual white, the fluids of the allantois and amnion and the membranes. The embryo and "remainder" were separately homogenised with measured volumes of water, using the apparatus of Potter and Elvehjem (1936). Only in the case of late embryos (18 days or older) did the time between removal from the freezing bath and the beginning of the analysis exceed thirty minutes. In their case it was found necessary to precede glass homogenisation by treatment for a few minutes in a Waring-type Blendor. Duplicate portions of all homogenates were analysed by the method given in the following sections.

2. PROCEDURE /

2. PROCEDURE FOR THE ESTIMATION OF NUCLEIC ACIDS AND PHOSPHOPROTEIN:

(i) Reagents.

Trichloroacetic acid 15% (w/v)

Ethanol A.R.

Ether A.R.

Ethanol-ether 2:3 (by volume)

Chloroform-methanol 1:1 (by volume)

Sodium hydroxide 1.00N

Hydrochloric-trichloroacetic acid 1.2N with respect to hydrochloric acid and 0.4N with respect to trichloroacetic acid. Prepared immediately before use by adding two volumes of 15% trichloroacetic acid to three volumes of 2N hydrochloric acid.

Reagents for phosphorus determination:

Ammonium molybdate, 8.3g. per 100ml. solution.

Amidol reagent, 1g. amidol and 20g. sodium bisulphite made to 100ml.

Perchloric acid 60%.

Reagents for the determination of inorganic phosphate:

Sodium hydroxide 2N, containing glycine 1M and sodium carbonate 0.2N.
Calcium chloride 10% (w/v)

Ammonia 1N, containing 1% calcium chloride.

Hydrochloric acid 2N.

(ii) Extraction /

(ii) Extraction of "Acid-soluble" and Lipoid Phosphorus.

One millilitre of homogenate was diluted to 12.5ml. with ice-cold water and shaken gently with 12.5ml. of ice-cold trichloroacetic acid in a glass-stoppered tube. After ten minutes the precipitate was separated by centrifugation and extracted a further three times in the same way. The residue was then shaken with 25ml. of ice-cold ethanol and centrifuged. In the case of "remainder" analyses, ethanol-ether was used for this extraction which in both cases was followed by two further extractions with ethanol-ether. The removal of lipoid phosphorus was completed by two extractions in each of which the precipitate was shaken with 10ml. of chloroform-methanol, the volume being increased to 25ml. by addition of ethanol-ether before centrifuging. The residue was then washed with ether and air-dried.

(iii) Incubation with Alkali and Separation of the Products.

The dried residue from these extractions (still in the original tube) was incubated at 37°C for 16 to 20 hours with a measured volume of normal sodium hydroxide. A total phosphorus determination was carried out on a portion of the incubate. A second aliquot was treated, after chilling, with an equal volume of the hydrochloric-trichloroacetic acid reagent and left for thirty minutes at /

at 0°C before centrifuging. The supernatant was analysed for total and inorganic phosphorus.

(iv) Determination of Total Phosphorus. (After Allen, 1940).

An aliquot of the solution to be analysed was pipetted into a bulbed tube containing 2.2ml. of perchloric acid and a small glass bead, and heated on a Kjeldahl rack until the digest was colourless and dense white fumes of perchloric acid ascended in the tube. After cooling, the digest was transferred with washings to a tube graduated at 20ml. and 25ml. After making to 20ml. with mixing, 2ml. of amidol reagent and 1ml. of ammonium molybdate were added, the tube being shaken after each addition. The volume was then made to 25ml. with a final shaking. The optical density of the blue solution was read after ten to twenty minutes against a blank prepared in the same way from the reagents only, in a Unicam S.P. 350 Spectrophotometer, set to a wavelength of 680 millimicrons. Phosphorus present was obtained by comparison with a calibration graph, prepared using a standard solution of potassium dihydrogen phosphate.

(v) Determination of Inorganic Phosphate.

This method was designed to estimate quantitatively amounts of inorganic phosphate from 5 to 40µg. in the acidified hydrolysate.

One /

One millilitre of the acid supernatant was diluted to approximately five times its volume in a centrifuge tube and 2N sodium hydroxide (containing glycine and sodium carbonate) added dropwise until the mixture was a definite pink to phenolphthalein. After addition of 1ml. of 10% calcium chloride and dilution to 10ml. with stirring, the tube was left for twenty minutes before centrifuging. The precipitate was washed once by suspension in 10ml. of 1N ammonia containing calcium chloride and dissolved in the minimum of 2N hydrochloric acid before transferring to a graduated tube for the estimation of phosphorus as in the method for total phosphorus.

(vi) Validity of the Methods Described.

Descriptions of experiments demonstrating the validity of the methods used will be found in Appendix I.

RESULTS AND DISCUSSION OF THE EMBRYO ANALYSES

In table I mean values for D.N.A.P. and R.N.A.P. of embryos of various ages from seven to nineteen days are given. These are plotted against incubation period in diagram 1. A list of individual values will be found in Appendix III. A comparison of the results with those of Novikoff and Potter (1948) shows a fair measure of agreement until the last few days, where the values seem to be lower than those of the American workers. This may be due in part to the very careful removal of spare yolk with its accompanying yolk sac membrane and to the fact that the embryos were in general slightly smaller than the average values given by Byerly (1932). The falling off in the rate of accumulation of D.N.A. and R.N.A. indicated by their analyses of late embryos is confirmed. Davidson and Leslie (1951) have, since this part of the work was completed, published values for the nucleic acid content of various organs of the chick embryo and have shown a decline in the rate of synthesis of nucleic acids in liver and heart during the last few days of incubation. The results given here suggest that the phenomenon may be more general.

RESULTS /

TABLE I.

Average Values for Nucleic Acid Phosphorus in Embryos of Different Ages

Embryo Age Days	Deoxy-ribonucleic Acid Phosphorus mg.	Ribonucleic Acid Phosphorus mg.
8	0.188	0.342
9	0.328	0.584
11	0.790	1.46
13	1.49	2.86
15	2.49	4.39
16	2.67	4.48
18	3.26	4.93
19	3.24	5.37

RESULTS AND DISCUSSION OF THE "REMAINDER" ANALYSES

Mean values for the phosphoprotein phosphorus of "remainders" of eggs at varicus stages of incubation are given in table II and plotted against incubation time in diagram 2. Values for individual eggs will be found in Appendix III.

Diagram 3 was obtained by plotting the negative slope of the smooth curve in diagram 2 against time. From diagram 3 it will be seen that the maximum rate of disappearance of phosphoprotein phosphorus occurs about the 13th or 14th day of incubation, the rate being then 3mg. per diem. The fact that the rate of phosphoprotein disappearance in the first seven days is very small, makes it appear unlikely that the yolk itself contains enzymes capable of breaking down phosphoprotein. In diagram 4, Byerly's figures (1932) for the wet weight of the yolk sac membrane are plotted against time of incubation. A certain similarity exists between this curve (4) and that in diagram 3 and would seem to indicate that the growth of this membrane plays a prominent part in determining the rate of phosphoprotein disappearance. The results obtained will be discussed more fully when the enzymic equipment of the yolk sac membrane has been described.

It is perhaps apposite at this point to re-

emphasise /

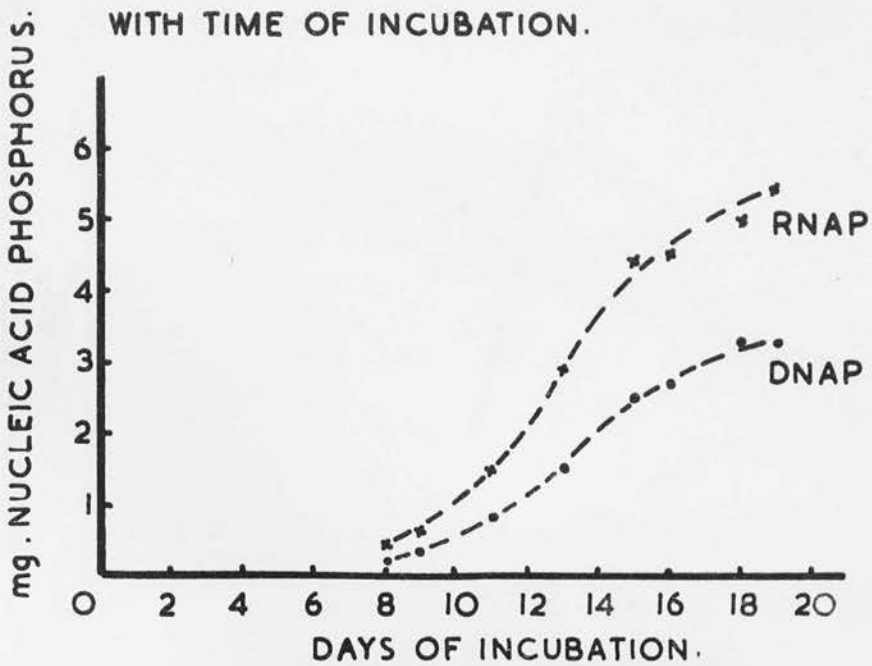
TABLE II.

Phosphoprotein Phosphorus in the "Remainder" at Various Stages of Incubation

Days of Incubation	Phosphoprotein Phosphorus mg.	Number of Eggs Analysed
0	27.8	18
7	25.7	5
8	26.2	5
9	26.1	6
10	26.1	13
11	24.3	9
13	18.2	9
14	15.8	6
16	11.8	9
17	9.6	14
18	8.0	8
19	6.1	7
20-21	5.3	4

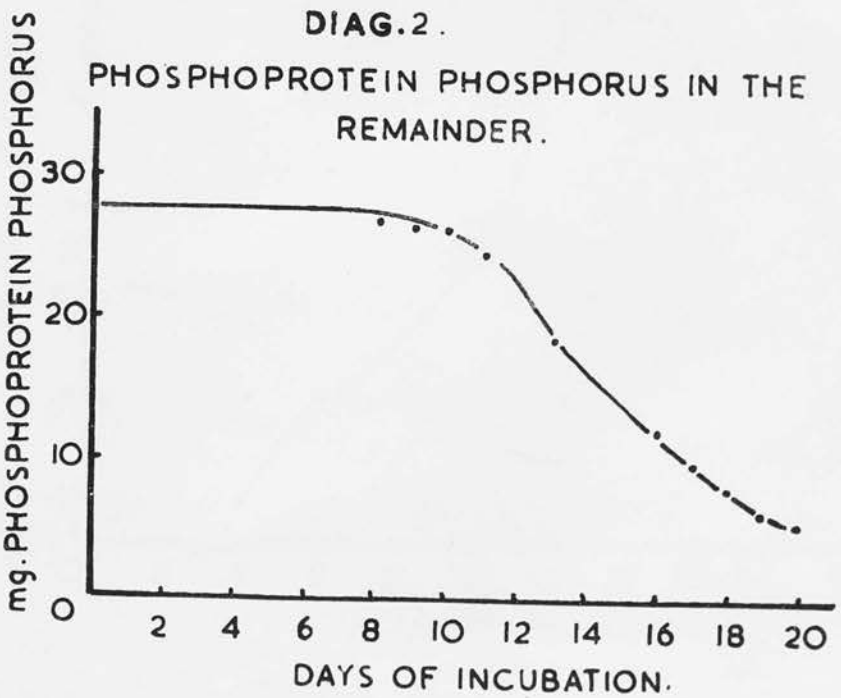
DIAG. 1.

VARIATION OF NUCLEIC ACID PHOSPHORUS WITH TIME OF INCUBATION.



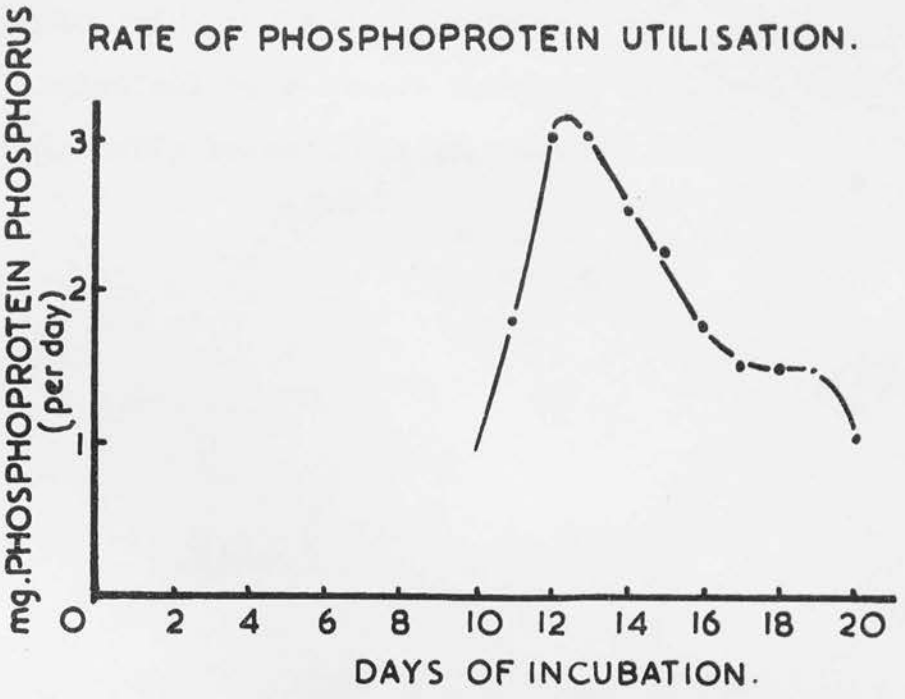
DIAG. 2.

PHOSPHOPROTEIN PHOSPHORUS IN THE REMAINDER.

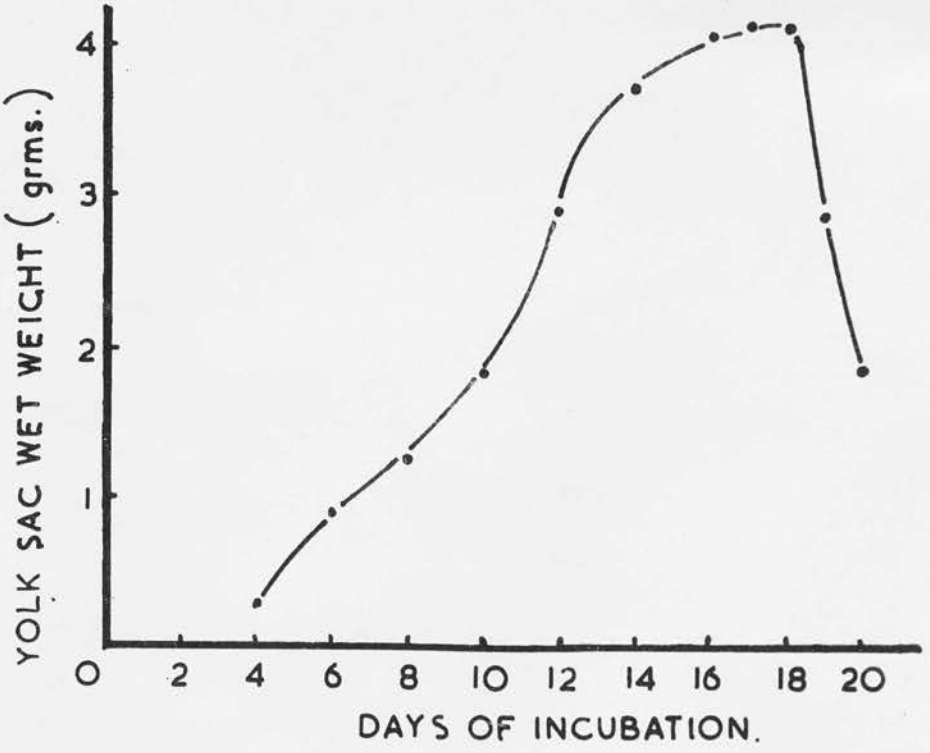


DIAG..3.

RATE OF PHOSPHOPROTEIN UTILISATION.



DIAG. 4.



emphasise that the simultaneous determination of the nucleic acids and phosphoproteins was purely a matter of analytical convenience and that no direct metabolic relationship between the two was suspected.

PART II

THE SITE OF PHOSPHOPROTEIN BREAKDOWN

INTRODUCTION

In Part I of this thesis it was shown that the yolk of an unincubated egg contains about 28mg. of phosphoprotein phosphorus and at the end of incubation only some 6mg. remain in the spare yolk; the amount of phosphoprotein phosphorus in the embryo (0.3mg. at hatching) would account for only a very small part of the phosphoprotein lost by the yolk.

The work to be described here was designed to trace where the phosphoprotein was being metabolised. Three major possibilities existed:

1) Phosphoprotein might be transferred intact to the embryo after absorption by the yolk sac membrane and metabolised in one or more of the embryonic organs.

2) Phosphoprotein might be broken down in the yolk either by enzymes present in the fresh yolk or by enzymes secreted by the yolk sac membrane.

3) Phosphoprotein might be absorbed intact into the yolk sac membrane and be metabolised there.

These possibilities were open to experimental examination. If the first were correct, the blood leaving the vascular plexus of the yolk sac should contain phosphoprotein phosphorus. The second and third possibilities might be tested by incubating portions /

portions of yolk alone or yolk and yolk sac membrane, and examining the incubates for phosphoprotein breakdown, using the technique of analysis developed in Part I.

EXPERIMENTAL

1. ANALYSIS OF BLOOD LEAVING THE YOLK SAC:

(i) Experimental method.

Twelve eggs which had been incubated for fourteen days were frozen hard and the blood vessels leading from the yolk sac excised. These, after weighing, were homogenised in an equal volume of water and 1.0ml. samples of homogenate taken for phosphoprotein analysis by the method used for embryo homogenates (p. 11). The experiment was repeated.

(ii) Results and Discussion.

In the two experiments performed, values of ten and twelve microgrammes of phosphoprotein phosphorus per gramme of blood vessel were obtained. The values are of the order of the amount of acid-soluble and lipoid phosphorus left in the residue from the extraction procedure used. It can be concluded that if phosphoprotein is carried in the blood leaving the yolk sac, it is at a lower concentration than can be detected by the methods available. To check that the method was suitable for the determination of phosphoprotein in blood, it was applied to the analysis of blood from laying hens and with it the observations of

Laskowski /

Laskowski (1935) of the existence of phosphoprotein there, were confirmed.

2. INCUBATION EXPERIMENTS WITH YOLK AND YOLK SAC MEMBRANE HOMOGENATES:

The experiments in this section were designed to find out if the yolk or yolk sac membrane contained enzymes capable of metabolising phosphoprotein.

The first series of experiments was performed on fresh yolk. It was thought that, if appreciable metabolism of phosphoprotein occurred in the yolk due to enzymes present there in the fresh egg, then on incubating portions of yolk material over an extended period one might be able to demonstrate phosphoprotein metabolism by comparing the quantity of phosphoprotein phosphorus in aliquots of yolk material before and after such incubation. Extending this idea, it was decided to analyse the aliquots of yolk material before and after incubation by a method which would estimate the inorganic, organic acid-soluble, lipoid, nucleic acid and phosphoprotein phosphorus on single aliquots of yolk material. Values obtained were expressed as percentages of the total phosphorus present in the aliquots. Phosphoprotein breakdown would thus be reflected as a decrease in the percentage of phosphoprotein phosphorus in yolk after incubation. The method /

method was independent of the actual amount of yolk material taken, as a value for the total phosphorus was obtained by summation of the values for total acid-soluble, lipid solvent soluble and residual phosphorus. The more complete analysis would indicate if other groups were being metabolised and, if phosphoprotein alone were being metabolised, whether it was giving inorganic or organic acid-soluble phosphorus.

Experiments of this type were performed with fresh yolk and with fresh yolk homogenates. It was felt that the yolk might contain enzymes capable of metabolising phosphoprotein, which were in some way prevented from acting in the intact yolk, but which might be released by homogenisation. Harris (1946) had found this state of affairs in the yolk of frogs' eggs, i.e. an enzyme capable of metabolising phosphoprotein existing in the yolk, but becoming active only on homogenisation.

The experiments performed in this section with yolks from fresh eggs suggested that the fresh yolk of the hen's egg was inactive towards phosphoprotein or any of the other groups estimated.

A second possibility in the metabolism of phosphoprotein in the system was that the material was metabolised by enzymes secreted into the yolk by the yolk sac membrane. If this occurred, one would expect that, /

that, on removing yolk from an egg in which the yolk sac membrane was developed, the yolk on incubation alone would undergo some further metabolism with respect to phosphoprotein.

Residual yolks were therefore removed from eggs at various stages of incubation and experiments carried out in the same way as the experiments with fresh yolk. Portions of homogenised residual yolk were analysed directly and the distribution of phosphorus among the groups, inorganic, acid-soluble, lipoid, nucleic acid and phosphoprotein, determined. Other portions were incubated for forty-eight hours and the phosphorus distribution again found. It was assumed that homogenisation would not seriously disturb the pH of the yolk.

A third group of possibilities of extra-embryonic metabolism of phosphoprotein metabolism was centred round the yolk sac membrane. The yolk sac membrane might absorb the phosphoprotein and metabolise it intracellularly or might cause a highly localised extracellular digestion by secretion of enzymes which, by adsorption or slowness of diffusion, remain close to the surface of the membrane. If any of these mechanisms were operative, one would expect to be able to demonstrate changes in phosphorus distribution on incubating /

incubating homogenates of yolk sac membrane with adherent yolk. Experiments similar to those done on fresh and residual yolks were therefore performed with material of this type.

(i) Experimental.

In experiments with fresh eggs the shell was split across and the white separated as far as possible from the yolk manually. Traces of white remaining on the vitelline membrane were removed by washing with fairly large quantities of 1% sodium chloride. The vitelline membrane was ruptured and removed by filtration through sterile gauze. The yolk was then pipetted directly or, in some cases, after treatment in a glass homogeniser into a series of sterile stoppered tubes. Two or three of these were used for immediate analysis, the others being placed in an incubator maintained at 37°C for 48 hours before analysis.

Incubated eggs were broken into a clean porcelain basin, the membranes of the allantois and amnion ruptured and the fluids with residual white washed away with 1% sodium chloride solution. The ruptured membranes were then picked off with forceps, leaving only the embryo attached to the yolk sac. After further washing, the yolk sac membrane was severed near /

near the point of attachment to the embryo and the embryo rejected. The yolk sac membrane was then drained of yolk as far as possible. The yolk drained off and yolk sac membrane with adhering yolk were separately treated in sterile glass homogenisers. Incubation and analysis were carried out as in experiments with fresh yolk.

A few experiments were also performed with eggs which, after incubation, were frozen hard in acetone-solid carbon dioxide. In these, the yolk and yolk sac membrane were separated as far as possible by simple dissection before homogenisation.

(ii) Analysis.

The material in the tubes was subjected to the extraction procedure described on p.11 for "Remainder" homogenates. Nucleic acid phosphorus and phospho-protein phosphorus were estimated in the usual way. Inorganic phosphate was determined using the method for inorganic phosphate previously described (p.13) and total acid-soluble phosphorus by Allen's method (p.13); both on aliquots of the first trichloroacetic acid extract. For the determination of lipoid phosphorus, the first four lipid solvent extracts were made to a volume of 100ml. and an aliquot taken which, after /

after evaporation to dryness in a bulb tube and digestion with perchloric acid, was determined by the standard technique.

(iii) Results and Discussion.

Results of experiments of the type described are given in tables III, IV, V and VI for yolk, and membrane with yolk, from eggs at various stages of incubation. Results are expressed as percentage of total phosphorus present (calculated from the values for total acid soluble, lipoid, nucleic acid and phosphoprotein phosphorus). This eliminates the error due to non--reproduceability of pipetting of undiluted yolk homogenates. The absolute values per ml. of homogenate would not be values for yolk due to volume changes during the washing period. Only values for inorganic and total acid-soluble phosphorus are presented, the changes observed in the amounts of lipoid, nucleic acid and phosphoprotein phosphorus in all experiments being within the limits of accuracy of the analytical methods employed.

Experiments in which yolk was incubated alone showed no significant change in the amounts of total acid-soluble phosphorus or inorganic phosphate through incubation. In experiments in which homogenised yolk sac /

TABLE III.a.

First Experiment

Before Incubation		After Incubation	
* Total Acid-soluble Phosphorus	Inorganic Phosphorus	Total Acid-soluble Phosphorus	Inorganic Phosphorus
4.3	4.0	4.4	3.9
4.8	4.3	4.2	4.1
3.8	3.7	4.5	3.9
4.3	4.0	4.4	4.0

TABLE III.b.

Second Experiment

Before Incubation		After Incubation	
* Total Acid-soluble Phosphorus	Inorganic Phosphorus	Total Acid-soluble Phosphorus	Inorganic Phosphorus
3.8	3.6	3.6	3.3
4.2	-	3.8	3.4
4.0	3.7	3.9	3.4
4.0	3.6(5)	3.8	3.4

Incubation of Fresh Yolk for 48 Hours

* All values expressed as percentage of total phosphorus.

TABLE IV.a.

First Experiment

Before Incubation		After Incubation	
* Total Acid-soluble Phosphorus	Inorganic Phosphorus	Total Acid-soluble Phosphorus	Inorganic Phosphorus
4.07	3.45	4.10	3.58
4.01	3.55	3.80	3.42
4.04	3.50	3.95	3.50

TABLE IV.b.

Second Experiment

Before Incubation		After Incubation	
* Total Acid-soluble Phosphorus	Inorganic Phosphorus	Total Acid-soluble Phosphorus	Inorganic Phosphorus
4.25	3.55	4.25	3.60
4.15	3.45	4.40	3.70
4.20	3.50	4.33	3.65

Incubation of Homogenised Fresh Yolk for 48 Hours

* All values expressed as percentage of total phosphorus.

TABLE V.a.

First Experiment
17 day egg

Before Incubation		After Incubation	
* Total Acid-soluble Phosphorus	Inorganic Phosphorus	Total Acid-soluble Phosphorus	Inorganic Phosphorus
9.3	3.7	10.8	8.3
9.1	3.7	11.2	8.5
9.2	3.7	11.0	8.4

TABLE V.b.

Second Experiment
17 day egg

7.9	4.1	10.4	7.8
7.8	3.7	10.4	8.2
7.8(5)	3.9	10.4	8.0

TABLE V.c.

Third Experiment
14 day egg

8.2	3.6	12.2	8.8
8.4	3.5	12.2	8.7
8.3	3.5(5)	12.2	8.7(5)

Incubation of Yolk with Yolk Sac Membrane Homogenates

* All values expressed as percentage of total phosphorus.

TABLE VI.

Incubation of Residual Yolk

Yolk of 13 day egg

Before Incubation		After Incubation	
* Total Acid-soluble Phosphorus	Inorganic Phosphate	Total Acid-soluble Phosphorus	Inorganic Phosphate
4.66	3.95	4.53	3.81
4.61	3.80	4.76	4.02
4.64	3.88	4.65	3.92

Yolk of 18 day egg

Before Incubation		After Incubation	
* Total Acid-soluble Phosphorus	Inorganic Phosphate	Total Acid-soluble Phosphorus	Inorganic Phosphate
5.61	4.20	5.64	4.61
5.84	3.98	5.88	4.35
5.72	4.11	5.76	4.48

Yolk from 18 day egg

Before Incubation		After Incubation	
* Total Acid-soluble Phosphorus	Inorganic Phosphate	Total Acid-soluble Phosphorus	Inorganic Phosphate
5.88	3.36	6.31	4.79
5.90	3.46	6.40	4.65
5.89	3.41	6.35	4.72

* All values expressed as percentage of total phosphorus.

sac membrane was present, the values for total acid--soluble phosphorus and inorganic phosphate were significantly increased after incubation. The experiments show that one or more of the groups, lipoid, nucleic acid and phosphoprotein, is being degraded, but the analyses are insufficiently accurate to determine which. The conditions of pH and ionic strength in these homogenates of yolk sac membrane are in all probability different from the metabolic sites of the intact tissue.

3. THE EFFECT OF pH TIME AND INHIBITORS ON THE PRODUCTION OF ACID-SOLUBLE PHOSPHORUS IN HOMOGENATES OF YOLK SAC MEMBRANE WITH YOLK:

(i) Preliminary Investigation with a Wide Range Buffer.

Experiments in the previous section having shown a rise of total acid-soluble phosphorus on incubating yolk sac homogenates, it was decided to examine the effect of pH on this process, in the hope that conditions might be found under which this increase would be considerably intensified, so enabling its source to be traced. Portions of yolk-yolk sac membrane homogenate were incubated in the same wide range buffer adjusted with sodium hydroxide or hydrochloric acid to pHs from 4.0 to 9.6. Parallel experiments were carried out in presence of fluoride and /

and of cyanide. The effect of fluoride, an inhibitor for numerous enzymes dealing with phosphates, was tried as it might stop the final liberation of inorganic phosphate and lead to the accumulation of intermediate organic acid-soluble compounds. The effect of cyanide was tested to eliminate the synthesis of organic phosphates by possible oxidative processes in the homogenates.

a) Experimental Method: A buffer mixture, 0.05M with respect to each of the following substances, potassium hydrogen phthalate, sodium acetate and sodium tetraborate, and 0.03M with respect to veronal, was prepared. From this, a series of buffers of pH 4.0, 5.1, 6.3, 7.3, 8.4 and 9.6 were prepared by addition of concentrated hydrochloric acid or 10N sodium hydroxide; the pH being tested electrometrically (glass electrode).

These eggs, incubated for 14 days, were frozen solid in acetone-solid carbon dioxide and yolk sac membrane with some yolk cut out and pooled for glass homogenisation in an equal volume of water. Three series of tubes were prepared. The first series contained 1ml. of homogenate, 3ml. of one of the buffers prepared above and 1ml. of water. The second series had 1ml. of homogenate, 3ml. of buffer and 1ml. of /

of 0.5M sodium fluoride and the third series 1ml. of homogenate, 3ml. of buffer and 1ml. of 0.005M potassium cyanide. The tubes were all placed in an incubator at 37°C for 48 hours, before analysis in the manner described previously for inorganic, acid-soluble, lipoid, nucleic acid and phosphoprotein phosphorus. Three one millilitre portions of homogenate were analysed directly in the same way to obtain the levels of these groups before incubation.

b) Results and Discussion: The values obtained for inorganic, acid-soluble, lipoid, nucleic acid and phosphoprotein phosphorus before and after incubation are given in table VII.

The most striking change in this experiment is at pH 4.0, where after incubation the phosphoprotein phosphorus has dropped to half its initial value; the inorganic phosphate having risen correspondingly. It seems probable that the rise in inorganic phosphate is attributable to the breakdown of phosphoprotein, although the alternative explanation that phosphoprotein is being broken down to organic acid-soluble phosphate, while original organic acid-soluble phosphates are being split to yield inorganic phosphate, exists. Both the disappearance of phosphoprotein and the rise in inorganic phosphate are prevented by fluoride.

At /

TABLE VII.

The Effect of pH on Yolk-Yolk Sac Incubates

Inhibitor	pH	Total Acid-soluble Phosphorus	Organic Acid-soluble Phosphorus	Inorganic P.	Lipoid P.	Nucleic Acid P.	Phospho-protein P.
Before Incubation							
-	-	11.1	6.1	5.0	72.8	3.6	12.4
-	-	10.8	5.7	5.1	73.6	4.0	12.4
-	-	11.0	6.2	4.8	72.8	3.7	12.3
-	Mean	11.0	6.0	5.0	73.1	3.8	12.4
After Incubation at 37°C for 48 Hours							
-		20.4	8.1	12.3	72.8	1.4	5.7
Cyanide	4.0	21.2	8.2	13.0	70.8	1.9	6.3
Fluoride		15.0	7.1	7.9	72.2	2.4	10.2
-		15.5	5.5	10.0	70.2	2.6	11.5
Cyanide	5.1	13.8	5.4	8.4	71.5	2.4	12.2
Fluoride		14.3	7.1	7.2	71.6	3.2	11.0
-		14.2	6.4	7.8	71.3	2.6	12.2
Cyanide	6.3	14.0	6.8	7.2	71.5	1.5	12.8
Fluoride		12.6	5.4	7.2	72.5	4.7	10.1
-		14.2	6.4	7.8	71.3	2.5	12.2
Cyanide	7.3	14.2	7.3	6.9	71.0	2.0	12.8
Fluoride		12.6	5.3	7.3	72.1	4.3	10.9
-		13.6	7.1	6.5	71.5	3.4	11.8
Cyanide	8.4	13.7	8.4	5.3	71.2	2.0	12.8
Fluoride		14.0	6.9	7.1	69.2	5.1	11.5
-		13.4	7.9	5.5	72.0	3.4	11.0
Cyanide	9.6	13.8	9.0	4.8	71.6	2.0	12.5
Fluoride		13.4	6.2	7.2	69.6	4.9	11.4

At the lower pH range, 4.0 to 7.3, there appears to be a decrease in nucleic acid phosphorus after incubation. Lipoid phosphorus values after incubation are slightly lower than the original value over most of the range investigated.

The presence of cyanide appears to make little difference to the changes observed.

(ii) The pH Optimum for Phosphoprotein Breakdown.

The previous experiment having shown extensive breakdown of phosphoprotein on incubating yolk sac homogenates at pH, it was decided to carry out a similar experiment over a narrower pH range to include the optimum. The main object of this experiment was to find out if the rise in inorganic phosphate would parallel the decrease in phosphoprotein phosphorus over a range of pH.

a) Method: A series of acetate buffers from pH 3.75 to 5.2 were prepared by mixing normal acetic acid with molar sodium acetate in proportions determined from the Henderson-Hasselbach equation, taking the pK of acetic acid as 4.75.

Yolk sac membranes with some adhering yolk were obtained by the method previously described on p. 24 from four eggs which had been incubated for 13 days. The /

The material was pooled and weighed before homogenisation with two volumes of water. 1ml. portions of homogenate were pipetted into a series of tubes containing 2ml. of acetate buffer and 2ml. of sodium chloride. These tubes were then incubated for 40 hours before analysis. Two one millilitre portions of homogenate were analysed directly. Inorganic, total acid-soluble, nucleic acid and phosphoprotein phosphorus were determined in this experiment.

b) Analysis: The method of analysis was similar to that used previously for incubation experiments, but all volumes in the extraction procedure for acid-soluble and lipoid phosphorus were reduced to two-fifths of those described on p. 12, making a final volume in each extraction of ten mililitres. The first trichloroacetic acid extraction was performed by adding 5ml. of ice-cold 20% trichloroacetic acid (the increased concentration to compensate for sodium acetate present) to the incubates. Analyses for acid-soluble and inorganic phosphate were done on 2ml. aliquots of this first extract. The dried residue from extraction was incubated with 5ml. of 1N sodium hydroxide. A 1ml. portion of the alkaline incubate was analysed for total phosphorus as nucleic acid and phosphoprotein. Inorganic phosphate, representing /

representing phosphoprotein phosphorus was determined on 2ml. of the acid supernatant from the incubate. All analyses were thus on corresponding fractions of the homogenate.

c) Results and Discussion: Values for acid-soluble, inorganic, nucleic acid and phosphoprotein phosphorus are given in table VIII. Changes in the values of these groups are given in table IX. Diagram 5 gives a diagrammatic representation of the results in table IX.

It will be seen that in diagram 5 the rise in inorganic phosphate obtained runs fairly closely to the curve for the amount of phosphoprotein phosphorus metabolised and there can be little doubt that phosphoprotein is being broken down to inorganic phosphate. The increase of the sum total acid-soluble + nucleic acid + phosphoprotein phosphorus must indicate some breakdown of lipoid material at the higher ranges studied.

The nucleic acid disappearance shows a minimum at pH 4.5 with maxima on either side. It is tempting to speculate that these represent maxima for D.N.A. and R.N.A. breakdown, but it is doubtful if the accuracy of the values based on the difference of two differences, and thus involving a quadruple error, would warrant /

TABLE VIII.

Distribution of Phosphorus before and after Incubation in Acetate Buffers of Various pH Values

Yolk-Yolk Sac Membrane Homogenate

	pH	Inorganic Phosphorus µg.P.	Organic Acid-soluble Phosphorus µg.P.	Nucleic Acid Phosphorus µg.P.	Phospho-protein Phosphorus µg.P.
Before Incubation		57	67.5	40	119
		57.5	66.5	44	116
		57.3	67	42	117.5
After Incubation	3.75	79.5	70.5	28	97
	4.2	114	71	28	72
	4.5	132.5	61.5	35	57.5
	4.5	133	62	36	56.5
	4.8	141.5	88.5	19	46.5
	5.2	135	83	21	61

TABLE IX.

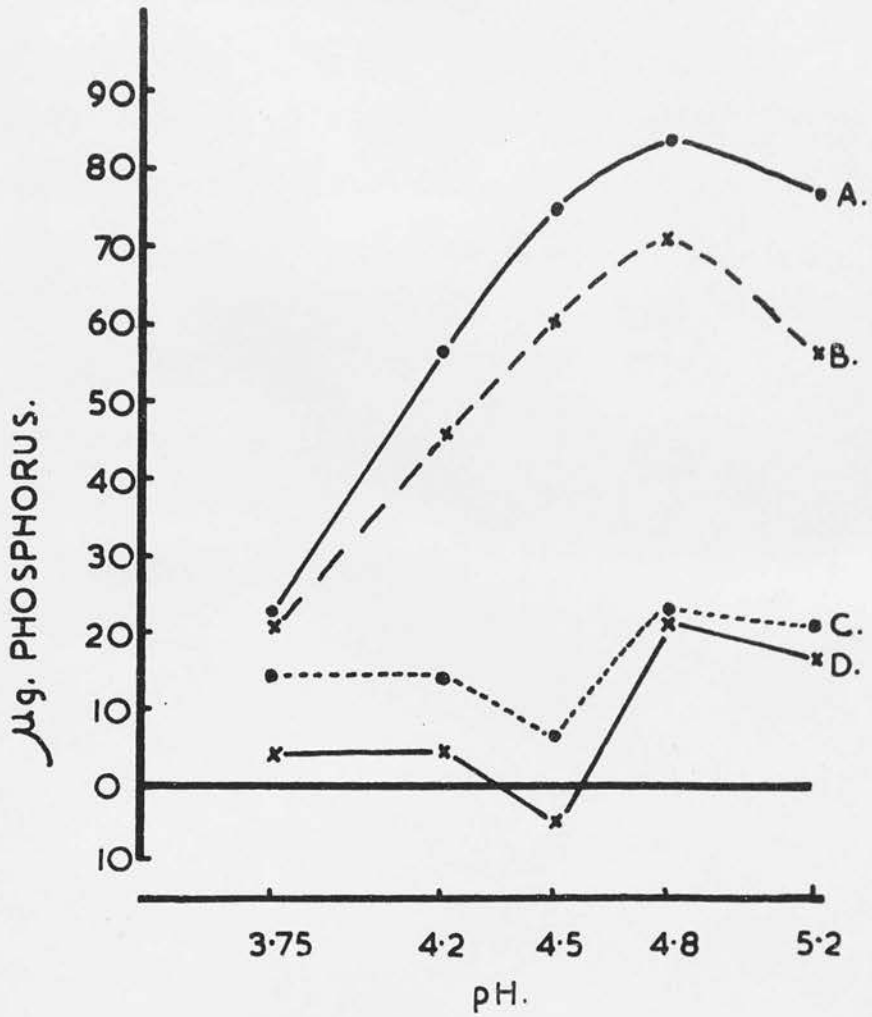
Changes in Amounts of Phosphorus in Various Groups of Phosphorus-containing Compounds after Incubation.

pH	Change in Amount of Inorganic Phosphorus µg.P.	Change in Amount of Phospho-protein Phosphorus µg.P.	Change in Amount of Organic Acid-soluble Phosphorus µg.P.	Change in Amount of Nucleic Acid Phosphorus µg.P.
3.75	22.(2)	-20.(5)	3.(5)	-14
4.2	56.(7)	-45.(5)	4.(0)	-14
4.5	75.(2)	-60.(0)	-5.(5)	- 7
4.5	75.(7)	-61.(0)	-5.(0)	- 6
4.8	84.(2)	-71.(0)	21.(5)	-23
5.2	77.(7)	-56.(5)	16.(0)	-21

Negative sign indicates decrease.

DIAG.5.

EFFECT OF pH. ON INCUBATION OF YOLK-YOLK SAC MEMBRANE HOMOGENATES



A. INCREASE IN INORGANIC PHOSPHORUS.
B. DECREASE IN PHOSPHOPROTEIN PHOSPHORUS.
C. DECREASE IN NUCLEIC ACID PHOSPHORUS.
D. INCREASE IN ORGANIC-ACID SOLUBLE PHOSPHORUS.

warrant this.

The actual value for the pH optimum for phosphoprotein breakdown is interesting in that it coincides with the value obtained by Harris (1946) for the phosphoprotein phosphorus of ripe yolk frog eggs. It is, however, fairly close to the figure of 4.0 given for the optimum of the proteinase of the yolk sac membrane (Borger and Peters 1933) and the values obtained for the acid phosphatase of the yolk sac membrane (Part III of this thesis) and so appears equally consistent with specific phosphoprotein phosphatase action, or with proteolysis followed by phosphatase activity as mechanism for the liberation of inorganic phosphate from phosphoproteins.

(iii) The Effect of Time of Incubation on Phosphoprotein Breakdown at pH 4.5.

The ability of yolk sac homogenates to liberate inorganic phosphate from phosphoprotein having been established, it was decided to investigate the effect of time of incubation on the process. It was felt that this might give some information on its nature. For the experiment a pH of 4.5 was chosen as giving least complication from nucleic acid breakdown.

a) Method: Nine grammes of yolk sac membrane and yolk obtained /

obtained by the standard method, were homogenised in 60ml. of 1M acetate buffer, pH 4.5. 3ml. of the resulting homogenate were pipetted into a series of tubes containing 2ml. of sodium chloride. After incubation for various times of from 0 to 72 hours at 37°C, the contents were analysed by the method given for the preceding experiment.

b) Results and Discussion: The values obtained for inorganic, acid-soluble, nucleic acid and phospho-protein phosphorus are given in table X and represented diagrammatically in diagram 6. It will be seen that the values obtained for organic acid-soluble phosphorus do not change significantly over the course of the experiment. This indicates that, if there is any breakdown of phosphoprotein to organic acid-soluble phosphates before the splitting off of inorganic phosphate, the latter process must be very rapid. The rise in the value of the sum of the groups estimated (organic acid-soluble + inorganic + nucleic acid + phosphoproteins) must indicate some ability of these homogenates to attack phospholipids. With the exception of the single analysis at 9 hours, the nucleic acid phosphorus values show a decrease with time. It seems likely that the analysis for phosphoprotein phosphorus at 9 hours was low, resulting in /

TABLE X.

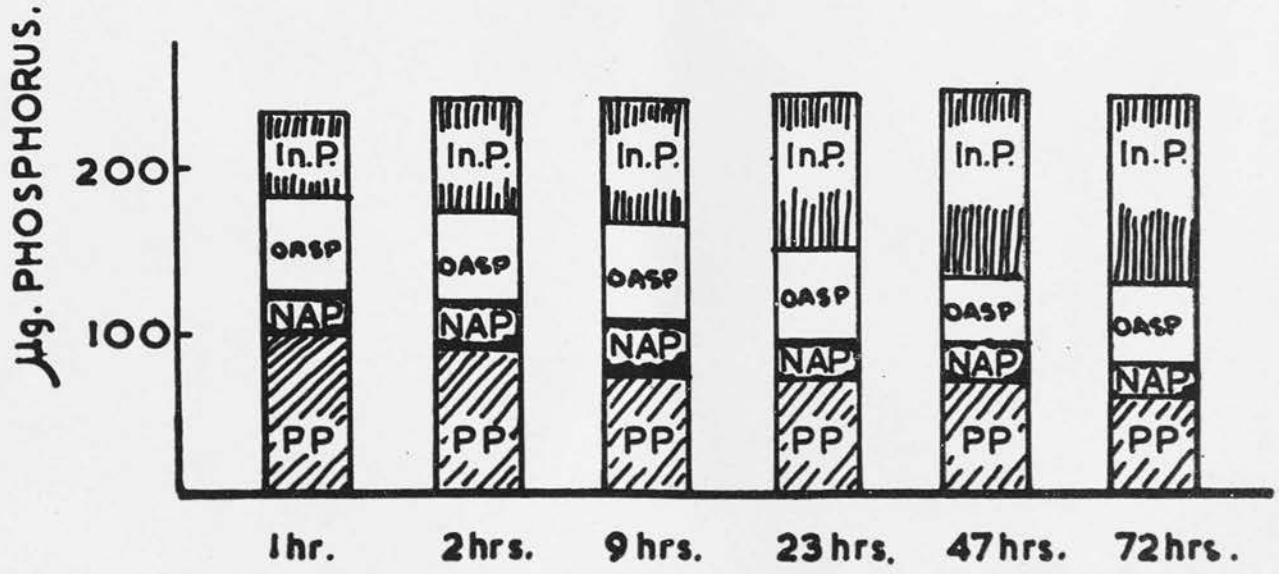
The Effect of Time of Incubation on Phosphorus Distribution in a Yolk-Yolk Sac Membrane Homogenate at pH 4.5.

Time Hours	Inorganic Phosphorus µg. P.	Organic Acid-soluble Phosphorus µg. P.	Nucleic Acid Phosphorus µg. P.	Phospho-protein Phosphorus µg. P.
0	49	58.5	27	98
2	65	59	28	90
9	75	60	33	73 *
23	96	53.5	25	70
47	115	40.5	25	67.5
72	117	47.0	20	60

* Comment in text.

DIAG. 6.

THE EFFECT OF TIME OF INCUBATION ON NON-LIPOID PHOSPHORUS DISTRIBUTION IN A YOLK-YOLK SAC MEMBRANE HOMOGENATE AT pH. 4·5 .



in a high value for nucleic acid phosphorus which is obtained by difference between the total residual phosphorus after solvent extraction and the inorganic phosphorus. This seems to be confirmed by the fact that the change in phosphoprotein phosphorus was greater in the period 2 to 8 hours than the increase in inorganic phosphorus - an observation contrary to what was observed in other experiments of this type.

4. THE LOCALISATION OF PHOSPHOPROTEIN BREAKDOWN:

(i) Comparison of Homogenates with and without Membrane.

It was necessary at this stage to examine whether the ability to break down phosphoprotein resided with the yolk sac membrane or was in the residual yolk. A comparison of the abilities of a residual yolk homogenate and homogenate containing yolk sac membrane was therefore carried out at pH 4.5.

a) Method: A fourteen day egg was frozen hard in acetone-solid carbon dioxide. Portions of residual yolk without membrane and yolk sac membrane with some yolk were excised with the scalpel, and after weighing were separately homogenised in the all glass apparatus with an equal volume of water. One millilitre portions of the homogenates were pipetted into tubes containing 4ml. of 0.75M acetate buffer at pH 4.5.

Two /

Two tubes were taken for analysis before, and two after 48 hours' incubation at 37°C, for each type of homogenate. Analysis was by the method used in the pH optimum and time dependence experiments described on p. 31.

b) Results and Discussion: Table XI and diagram 7 give the analytical values obtained. The ability to break down phosphoprotein and nucleic acids exists only in the homogenates containing yolk sac membrane, although the yolk homogenates show some slight ability to form inorganic phosphate from organic acid-soluble phosphorus.

(ii) Incubation of a Late Residual Yolk Homogenate at pH 4.5.

It appeared from the last experiment that the residual yolk of a fourteen day egg did not undergo any degradation of phosphoprotein to other types of phosphorus compound. It was decided to investigate if this inactivity of the residual yolk existed through the incubation period. The residual yolk of an egg after 18 days of incubation was therefore examined.

a) Method: An 18 day egg was frozen hard and residual yolk excised with the scalpel, care being taken to avoid contamination with membrane. The excised yolk was /

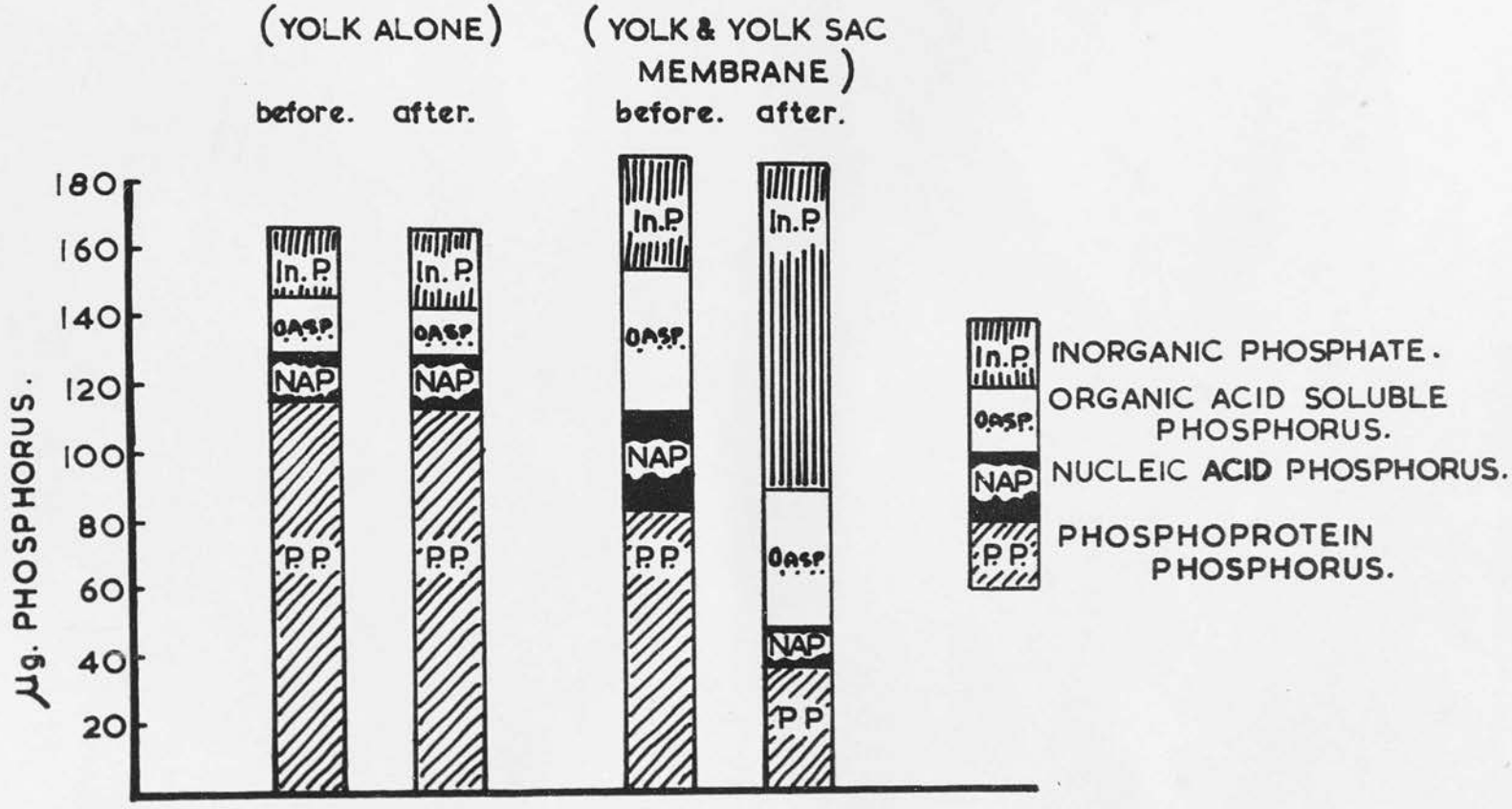
TABLE XI.

Comparison of the Effect of Incubation on a Residual Yolk and a Yolk with Yolk Sac Membrane Homogenate at pH 4.5

Residual Yolk Homogenate				
	Inorganic Phosphorus µg. P.	Organic Acid-soluble Phosphorus µg. P.	Nucleic Acid Phosphorus µg. P.	Phospho-protein Phosphorus µg. P.
Before Incubation	20.6	16.2	16.4	113.6
	20.0	16.4	12.0	116
After Incubation	23.0	12.6	15.2	112.8
	23.0	15.8	16.0	112
Residual Yolk with Membrane Homogenate				
	Inorganic Phosphorus µg. P.	Organic Acid-soluble Phosphorus µg. P.	Nucleic Acid Phosphorus µg. P.	Phospho-protein Phosphorus µg. P.
Before Incubation	32.8	41.2	30	82
	33.0	43.0	29	83
After Incubation	95.0	41.0	11	39
	96.0	40.8	10	36

DIAG.7.

COMPARISON OF THE EFFECT OF INCUBATION ON A RESIDUAL YOLK AND A YOLK WITH YOLK SAC MEMBRANE HOMOGENATE AT pH. 4·5 .



was homogenised in an equal volume of water and 1ml. portions pipetted into a series of six tubes. Two of these were analysed directly, two were incubated with buffer and two incubated with 4ml. of 0.75M acetate buffer, pH 4.5, for 48 hours before analysis. Analysis was as for the preceding experiments (p. 31).

b) Results and Discussion: The results of this experiment, given in table XII, show that some breakdown of late yolk phosphoprotein may occur extracellularly.

(iii) The Activity of the Early Yolk Sac Membrane.

As the experiments on the phosphoprotein remaining at various stages of incubation had shown only a small difference, 1.1mg. of phosphoprotein phosphorus, between the values for unincubated eggs and those at ten days, it was felt necessary to examine the yolk sac membrane at an early stage of incubation for the ability to metabolise phosphoprotein.

a) Method: The yolk sac membranes, with some yolk from two 7 day embryonated eggs, were obtained by the saline washing technique described on p. 24 and homogenised in an equal volume of water. 1ml. aliquots of the homogenate were pipetted into four tubes, each containing 2ml. of 1M acetate buffer, pH /

TABLE XII.

The Effect of Incubation on an 18 day Residual Yolk

	Inorganic Phosphate µg.	Organic Acid-soluble Phosphorus µg.	Nucleic Acid Phosphorus µg.	Phospho-protein Phosphorus µg.
Before Incubation	46	34.5	25	150
	52.5	31.0	25	151
After Incubation pH 4.5	56	30.5	15	145
	64	25.5	15	145
After Incubation No Buffer	63	31	20	146.5
	64	33	15	150

pH 4.5. Analyses before and after incubation were performed, as in the preceding experiments.

b) Results and Discussion: Table XIII and diagram 8 give the results of this experiment. The phosphoprotein phosphorus and ribonucleic acid phosphorus have fallen markedly during incubation. Inorganic phosphate has risen by an amount greater than the amount of phosphoprotein phosphorus lost, and indicates that other groups are being broken down to inorganic phosphate. Organic acid-soluble phosphorus increases by a small amount. The sum of the phosphorus in the groups estimated rises, indicating the conversion of phospholipid phosphorus to one of these groups.

The experiment shows that, at seven days, the yolk sac membrane has enzymes which attack phospholipids, phosphoproteins and ribonucleic acid groups, which together account for nearly all of the phosphorus of egg yolk.

(iv) Analysis of Yolk Sac Membrane.

It would appear that information on the question of intra- or extracellular metabolism of phosphoprotein might be obtained by analysis of portions of yolk sac membrane for phosphoprotein. If the yolk sac membrane contains phosphoprotein, then some degree of intracellular /

TABLE XIII.

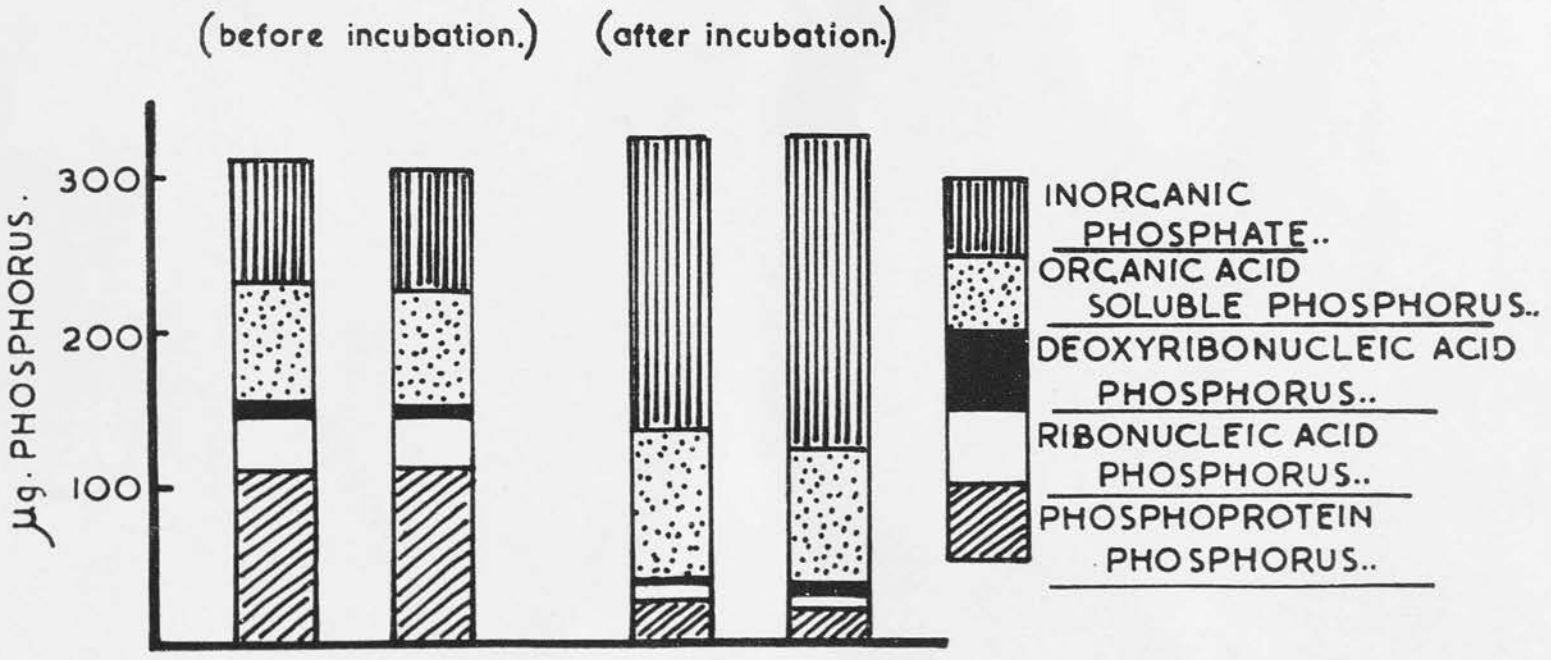
Incubation of a 7 day Yolk Sac Membrane Homogenate with some Yolk at pH 4.5 and 37° C for 48 hours.

Phosphorus Distribution Before and After Incubation.

	Inorganic Phosphorus µg. P.	Organic Acid-soluble Phosphorus µg. P.	Deoxy-ribonucleic Acid Phosphorus µg. P.	Ribonucleic Acid Phosphorus µg. P.	Phospho-protein Phosphorus µg. P.
Before Incubation	78	78	10	34	111
	78	78	3.5	35.5	111
After Incubation	190	100	0.5	11	26
	205	87	4.5	12.5	20.5

DIAG. 8.

NON-LIPOID PHOSPHORUS DISTRIBUTION BEFORE & AFTER INCUBATION
7 DAY YOLK-YOLK SAC HOMOGENATE.



cellular digestion must occur, although the possibility of extracellular metabolism occurring at the same time is not excluded.

The very considerable degree of convolution and the extreme fragility of the yolk sac membrane make the task of freeing it completely of adherent yolk almost impossible. It was felt that only at an early stage of incubation was there any hope of obtaining yolk sac membrane, free of attached yolk. Seven day yolk sac membranes were excised and washed in several changes of physiological saline. Portions of the washed material were examined histologically; other portions were homogenised and analysed for phosphoprotein phosphorus.

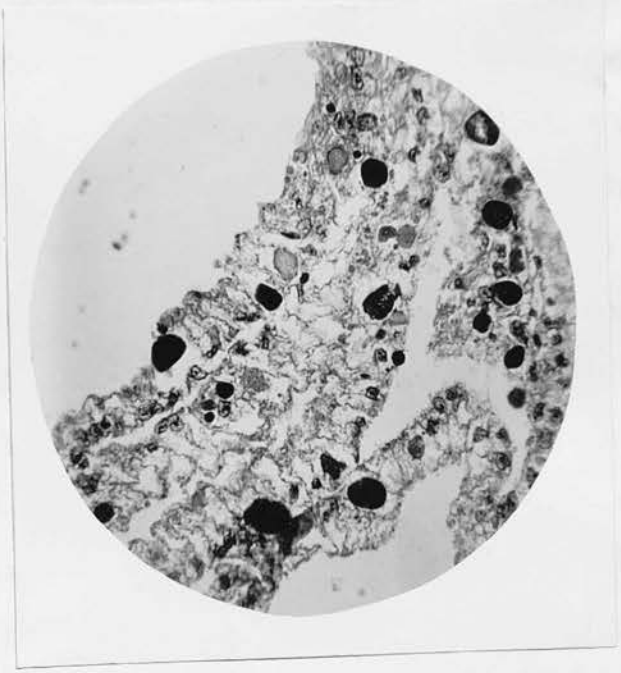
It was found that even with these seven day membranes, it was impossible to obtain a preparation wholly free from adherent yolk. Analysis of the washed yolk sac membrane showed some 0.4mg. of phosphoprotein phosphorus per gramme. If no phosphoprotein phosphorus were contained in the yolk sac membrane itself, this would indicate, taking the level of phosphoprotein phosphorus in the yolk as approximately 1mg. per gramme, that the material analysed consisted of some 40% of yolk. Examination of sections stained with haemotoxylin-eosin seems to indicate that the degree /

degree of contamination with yolk, of the material analysed, was well below this value. Photomicrograph (1) gives a typical area of the sections examined.

It would seem probable, therefore, that yolk sac membrane contains phosphoprotein, or that the phosphoprotein is considerably concentrated close to the yolk sac membrane. Of these alternatives, the former, occurrence of phosphoprotein within the cells of the yolk sac membrane, seems more likely. The experiment, although not affording anything like complete proof of the occurrence of phosphoprotein in the cells of the yolk sac membrane, and therefore of intracellular digestion, does point in that direction.

Photomicrograph (1)

Washed Yolk Sac Membrane Stained
Haemotoxylin-eosin
x 320



SUMMARY AND DISCUSSION

As outlined in the introduction to this section, three main possibilities appeared to exist for the metabolism of phosphoprotein in the embryonated system. The first of these was transport via the yolk sac membrane and its circulation to the embryo for metabolism; the second was absorption of the material by the yolk sac membrane, followed by metabolism within the cells of the membrane; and the third, extracellular digestion in the yolk by enzymes present there in the fresh egg, or secreted by the yolk sac membrane. The possibilities were tested experimentally. Analysis of the blood in the large vessels between the yolk sac and the embryo at the 13th day of incubation, when the disappearance of phosphoprotein phosphorus is maximal, failed to demonstrate phosphoprotein phosphorus in amounts greater than could be accounted for by the "blank" of the analytical method. Direct phosphoprotein transport to the embryo therefore seems improbable, unless the blood flow through the membrane system is so high that the level of phosphoprotein phosphorus present at any time is below the level of detectability. Attention was therefore concentrated on the possibility of extra-embryonic metabolism. Experiments /

Experiments were carried out in which portions of fresh yolk were incubated for 48 hours at 37°C and analysed. These experiments failed to show any change in the distribution of phosphorus between the groups, inorganic, organic acid-soluble, lipoid, nucleic acid and phosphoprotein phosphorus, and it was therefore concluded that if there was any activity of yolk enzymes on phosphorus compounds, it was not of quantitative importance. Experiments carried out in the same way with residual yolk gave no indication of metabolic activity, except with preparations from eggs in the terminal stages of development. Homogenates of yolk sac membrane with yolk showed some activity and were accordingly investigated over a range of pH.

It was found that at pH values around 5, there was considerable breakdown of phosphoprotein, this being accompanied by a rise in inorganic phosphate; the rise in inorganic phosphate running fairly close to, but always higher than the amount of phosphoprotein phosphorus disappearing. The homogenates also showed some ability to break down nucleic acids. The curve for disappearance of nucleic acid with variation of pH resembled to some degree the curve for production of acid soluble phosphorus. The sum of the materials estimated in this experiment, total acid-soluble, nucleic acid and phosphoprotein, showed an increase

at /

at pH values around 5 and it would seem, therefore, that there must have been some degradation of phospholipids under the conditions of the experiment. The number of changes observed in a system of this type makes the interpretation difficult, but fortunately the change in phosphoprotein phosphorus was considerably larger than those of organic acid-soluble, nucleic acid and lipoid phosphorus and the experiment clearly establishes the ability of yolk sac membrane homogenates to liberate the phosphorus of phosphoprotein as inorganic phosphate. An experiment in which the time of incubation of a yolk-yolk sac membrane homogenate was varied from 0 to 72 hours, again showed the rise in inorganic phosphate to run roughly parallel to the amount of phosphoprotein phosphorus lost. The activity of residual yolk at pH 4.5 (near the optimum for phosphoprotein breakdown with membrane homogenates) was indetectable at stages up to 15 or 16 days, but to exist, though at a low value, at 18 days.

It emerges from these experiments that a system exists, capable of metabolising phosphoprotein, and that at least until the terminal stages of incubation this system is closely associated with the yolk sac membrane. It seems plausible to assume that this system may be responsible for the metabolism of phosphoprotein /

phosphoprotein in vivo, in which case the process of utilisation of phosphoprotein may occur in two ways; the first of these, absorption of phosphoprotein into the yolk sac membrane, followed by intracellular metabolism; the second, extracellular digestion by enzymes secreted by the membrane but acting over a limited space close to the membrane.

When seven day yolk sac membranes were washed as far as possible, free from yolk and analysed, they appeared to contain more phosphoprotein than the amount of yolk contamination, as judged on histological examination, could account for. This affords evidence (although not conclusive proof) that phosphoprotein enters the cells of the yolk sac membrane.

From the facts detailed here, the following picture of phosphoprotein utilisation seems the most acceptable.

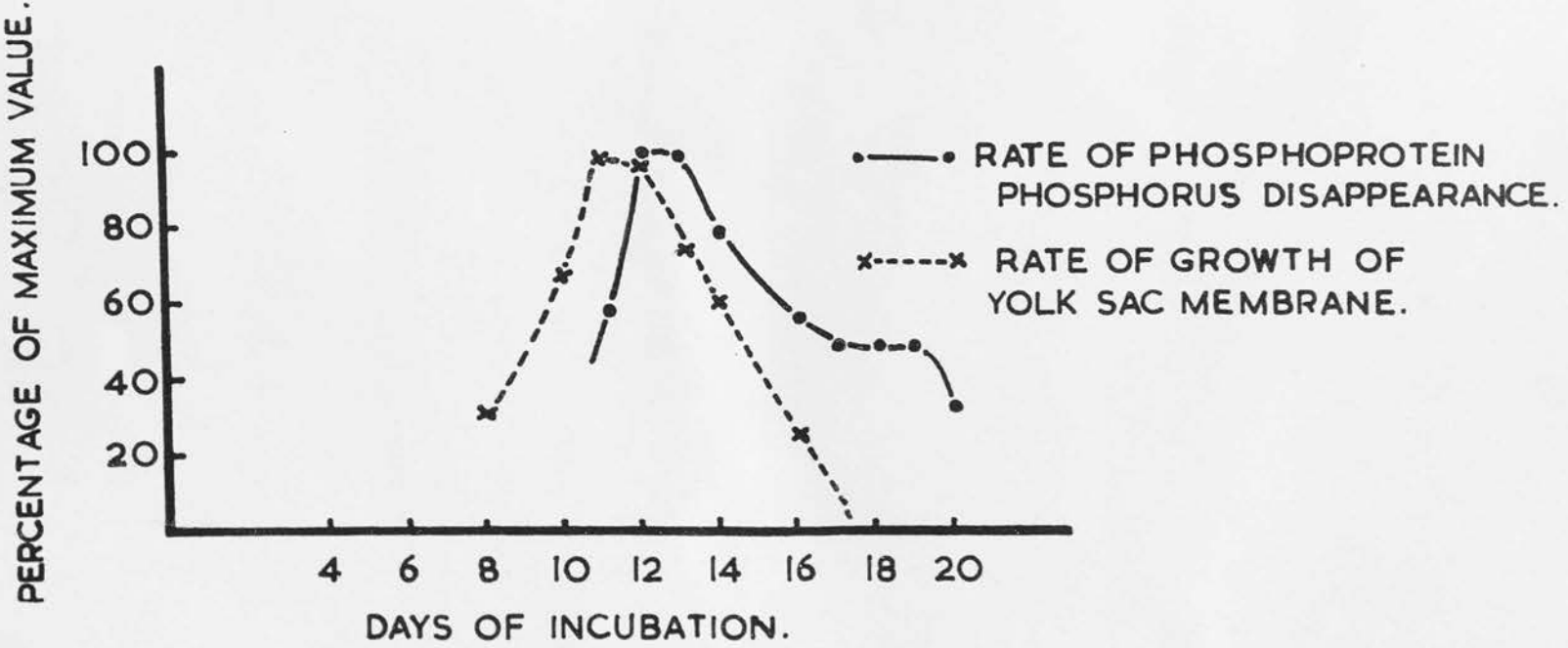
Until the last few days of incubation, the phosphoproteins of the yolk are absorbed directly into the cells lining the yolk sac membrane, to be attacked by enzymes existing there, with the liberation of inorganic phosphate which may presumably be used by the cells of the membrane or transported in the blood to the embryo. Towards the end of incubation, some degree of extracellular digestion occurs in the residual /

residual yolk, either by enzymes secreted by the cells of the membrane or released during the regression of the membrane.

If these ideas are correct, then one might expect some correlation between the rate of growth of the yolk sac membrane, which grows into the yolk, and the rate of utilisation of phosphoprotein phosphorus. In diagram 9 these two values are plotted as percentages of their maxima and it will be seen that a very distinct similarity exists while the membrane is growing, until about the 16th day, and that thereafter they diverge widely at the period when extracellular digestion begins.

DIAG. 9.

COMPARISON OF GROWTH RATE OF YOLK SAC MEMBRANE WITH RATE OF PHOSPHOPROTEIN PHOSPHORUS DISAPPEARANCE.



PART III

THE PHOSPHOMONOESTERASES OF THE YOLK SAC MEMBRANE

INTRODUCTION

The work reported in Part II of this thesis having shown that the yolk sac membrane can metabolise various types of phosphorus compounds, it was thought necessary at this point to carry out a preliminary exploration, using a standard assay procedure, for enzymes of the wide specificity acid and alkaline phosphomonoesterase type. As far as the author is aware no previous studies of this nature have been made on the membrane.

The titration studies of Lipmann(1933) on products obtained from yolk phosphoprotein, indicate that phosphate groups are singly esterified on the hydroxyls of the serine residues in the protein. Two possible methods of phosphoprotein breakdown might be, direct action of a phosphomonoesterase liberating inorganic phosphate, or proteolysis to phosphopeptides or serine phosphate followed by phosphomonoesterase action.

Preliminary experiments showed the yolk sac membrane to contain both an acid and an alkaline phosphatase. The pH optimum for the phosphoprotein to inorganic phosphate change in yolk sac homogenates being 4.8, attention was concentrated on the acid phosphomonoesterase as the more likely participant in phosphoprotein metabolism. An attempt was therefore made /

made to purify the acid phosphatase and to test its activity on lipovitellin and products obtained by the action of proteolytic enzymes on the phosphoprotein. Estimations of the acid phosphatase activity of the membrane at various stages of development were carried out, in the hope that the activity might be correlated with the rate of disappearance of phosphorus compounds from the yolk and the requirements of the embryo.

EXPERIMENTAL

1. THE METHOS OF ENZYME ASSAY:

For the enzyme assay, the method of King and Armstrong (1934), with some minor modifications as used by Kerr et al. (1948), was chosen, being rapid and sensitive. In this method, enzyme activity is measured by the amount of phenol liberated from phenyl phosphate on incubation for a fixed time. The phenol is determined colorimetrically using the reagent of Folin and Ciocalteu (1927).

(i) Reagents.

Substrate solution: Disodium phenyl phosphate, adjusted, if necessary, to the pH used in the assay by addition of acetic acid.

Folin-Ciocalteu reagent:
a) Stock solution (B.D.H.)
b) Stock solution diluted 1:5 shortly before use.

1N Sodium carbonate solution.

(ii) Procedure.

Three centrifuge tubes were used in each assay. The first of these was prepared with 0.2ml. of the material to be tested, 0.2ml. of phenyl phosphate solution and 0.4ml. of buffer. In the second, 0.2ml. of /

of water replaced the substrate solution and in the third, the enzyme preparation was replaced by 0.2ml. of water. The second and third tubes were necessary for preparation "blanks" and substrate "blanks" respectively. The phenyl phosphate solution was added after the tubes had been in a water bath at 37°C for five minutes, and the period of hydrolysis timed from this addition. After the desired period of incubation 2ml. of 1:5 diluted Folin-Ciocalteu reagent were added to each tube, the contents mixed and the tube removed from the bath to stand for five minutes. The tubes were centrifuged for five minutes to pack the protein precipitate formed. 2.0ml. of the supernatant liquid were transferred to a tube containing 4.0ml. of 1N sodium carbonate solution, the contents mixed and colour-developed by incubation for twenty minutes at 37°C. The optical density of the solution was measured in the Unicam S.P. 350 Spectrophotometer set to a wavelength of 680 millimicrons. Phenol present was obtained by comparison with a calibration graph, prepared by taking known amounts of phenol in 0.8ml. of solution, treating with 2.0ml. of diluted Folin--Ciocalteu reagent and taking 2.0ml. of the resulting solution for colour development, as described for the enzyme assays.

Phenol /

Phenol liberated from phenyl phosphate by phosphatase was obtained by subtracting the sum of the phenol values for the second and third tubes from that of the first.

2. DEMONSTRATION OF PHOSPHOMONOESTERASES IN THE YOLK SAC MEMBRANE:

(i) The Activity of Yolk-Yolk Sac Membrane Homogenates.

The first experiment was designed to show whether yolk-yolk sac membrane homogenates contained "acid" and "alkaline" phosphomonoesterases.

a) Method: A series of buffers covering the pH range from 4.0 to 9.5 were prepared by addition of concentrated hydrochloric acid or sodium hydroxide solution to a mixed buffer, which was 0.05M with respect to potassium hydrogen phthalate, sodium acetate and sodium tetraborate, and 0.03M with respect to veronal.

Three eggs incubated for 14 days were frozen hard, dissected and the yolk sac membranes, with some yolk, homogenised in ten volumes of water. Assays of phenol liberated from phenyl phosphate at various pH values by 0.2ml. of the homogenate, in 30 minutes at 37° C, were made by the technique described previously (p. 48). The concentration of phenyl phosphate used was 0.04M.

b) Results and Discussion: The values given in table XIV are corrected for substrate and enzyme "blanks" and /

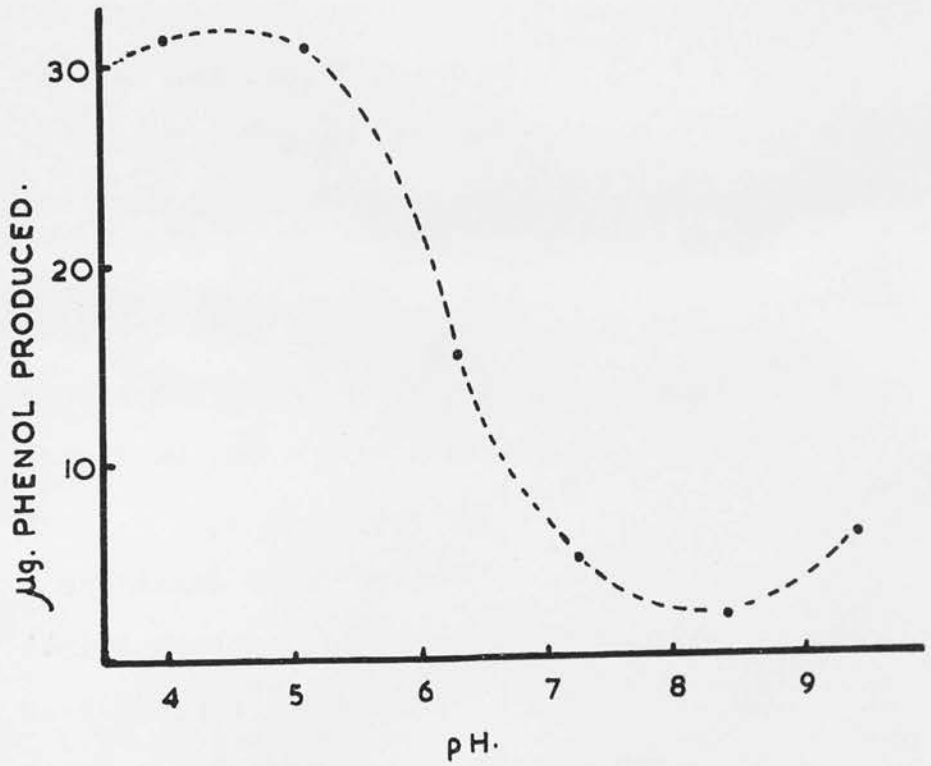
TABLE XIV.

Phenyl Phosphatase Activity at Various pH Values of a Yolk-Yolk Sac Membrane Homogenate

pH	Phenol liberated µg.	pH	Phenol liberated µg.
4.0	31.8	7.3	5.0
5.1	31.2	8.4	2.2
6.3	15.5	9.5	6.1

DIAG.10.

PHENYL PHOSPHATASE ACTIVITY AT VARIOUS pH.
VALUES OF A YOLK-YOLK SAC MEMBRANE HOMOGENATE.



and represent phenol liberated from phenyl phosphate by 0.2ml. of the dilute yolk-yolk sac membrane homogenate in 30 minutes. The experiment shows that such homogenates contain both an "acid" and an "alkaline" phosphomonoesterase.

(ii) Demonstration that the Phosphatases Investigated are Associated with the Yolk-Yolk Sac Membrane.

This experiment was designed as a comparison of the phosphatase activities of yolk sac membrane homogenates and yolk homogenates.

a) Method: Two 13 day incubated eggs were opened and the yolk sac isolated by the saline washing technique (p. 24). The yolk was then drained from the pierced yolk sac membrane, which was then washed with 1% sodium chloride. The yolk and yolk sac membranes were then separately homogenised in ten volumes of water. The resulting suspensions were tested for phosphatase activity at pH 4.5 and pH 9.2 by the technique described previously on p. 48. The buffer used at pH 4.5 was 0.4M acetate and at 9.2, 0.1M borate. 0.2ml. of 0.04 phenyl phosphate was used as substrate. The incubation period was 30 minutes.

b) Results /

b) Results and Discussion:

At pH 4.5

Phenol liberated by 0.2ml. dilute yolk sac membrane homogenate = 40.0µg.

Phenol liberated by 0.2ml. dilute residual yolk homogenate = 0.0µg.

At pH 9.2

Phenol liberated by 0.2ml. dilute yolk sac membrane homogenate = 2.5µg.

Phenol liberated by 0.2ml. dilute residual yolk homogenate = 0.1µg.

From these results it would appear that the enzymic activity rests only with the membrane.

(iii) Proof that the Phosphatase Activity of Yolk Sac Membrane Homogenates is not Accounted for by the Blood Present.

In the previous experiment the homogenate of yolk sac membrane used, unavoidably contained some blood from the membrane circulation. The possibility therefore existed that the activity of the homogenates was due to phosphatases from the blood present. Experiments were therefore carried out in which determinations of phosphatase activity were made on blood from the large vessels connecting the yolk sac to the embryo and on membrane homogenates. The activity of the homogenates due to the blood present in them was then /

then calculated using haem iron as a measure of blood contamination and compared with the experimental value for the homogenate. It was assumed that the yolk sac membrane contained little haem iron other than that of the haemoglobin of its circulation.

a) Experimental Procedure: Two 14 day eggs were frozen hard. Blood vessels from the yolk sac membrane circulation and portions of the membrane itself were excised, weighed and separately homogenised after addition of nine volumes of water.

The homogenates were analysed for haem iron by the method of Ramsay (1951). Estimations of phosphatase activity were carried out by the method described previously, using 0.2ml. of 0.04M phenyl phosphate, 0.2ml. of preparation and 0.4ml. of 0.4M acetate buffer pH 4.5 and 0.1M borate buffer pH 9.2. The incubation period was one hour.

b) Results: Analysis of First Egg

Haem iron content of blood homogenate	=	25.8ug./ml.
Haem iron content of membrane homogenate	=	1.0ug./ml.
Phenol liberated at pH 4.5 by blood homogenate	=	5.7ug.
Phenol liberated at pH 4.5 by membrane homogenate	=	46.9ug.
Percentage of phenol liberated by membrane homogenate at pH 4.5 accounted for by blood present	=	$\frac{1.0}{25.8} \times \frac{5.7}{46.9} \times 100$
	=	0.47%

Phenol liberated at pH 9.2 by blood homogenate = 7.8µg.

Phenol liberated at pH 9.2 by membrane homogenate = 3.5µg.

Percentage of phenol liberated by membrane homogenate at pH 9.2 accounted for by blood present

$$= \frac{1.0}{25.8} \times \frac{7.8}{3.8} \times 100$$

$$= \underline{8.0\%}$$

Analysis of Second Egg

Haem iron content of blood homogenate = 13.8µg./ml.

Haem iron content of membrane homogenate = 1.03µg./ml.

Phenol liberated at pH 4.5 by blood homogenate = 4.0µg.

Phenol liberated at pH 4.5 by membrane homogenate = 47.6µg.

Percentage of phenol liberated by membrane homogenate at pH 4.5 accounted for by blood present

$$= \frac{1.03}{13.8} \times \frac{4.0}{47.6} \times 100$$

$$= \underline{0.63\%}$$

Phenol liberated at pH 9.2 by blood homogenate = 4.2µg.

Phenol liberated at pH 9.2 by membrane homogenate = 3.6µg.

Percentage of phenol liberated by membrane homogenate at pH 9.2 accounted for by blood present

$$= \frac{1.03}{13.8} \times \frac{4.2}{3.6} \times 100$$

$$= \underline{8.7\%}$$

c) Discussion: /

c) Discussion: From these results it may be taken that the yolk sac membrane contains acid and alkaline phosphomonoesterases other than those of its circulation, and that in the case of the acid phosphomonoesterase contamination of membrane homogenates with blood from the villi will not appreciably alter the activities found.

3. SOME PROPERTIES OF THE ACID PHOSPHATASE OF THE YOLK SAC MEMBRANE:

It having been established that the yolk sac membrane contains an acid phosphomonoesterase, some experiments were carried out on its general properties with a view to finding suitable conditions for enzyme assay.

(i) The pH Optimum of the Enzyme.

In the preliminary experiment showing the existence of the enzyme in yolk sac homogenates, maximum activity was observed at pH 4-5 with the mixed buffer employed. Acetate-acetic acid buffers (pK acetic acid 4.75) appeared suitable for work in this region and the activity of membrane homogenates was accordingly tested over the effective range of buffers of this type.

a) Experimental Procedure: 0.4M sodium acetate-acetic acid /

acid buffers were prepared covering the pH interval 3.75 to 4.75.

The yolk sac membrane was excised from a frozen 16 day egg and homogenised in some twenty volumes of water. 0.2ml. portions of the resulting homogenate were incubated with 0.2ml. of 0.04M phenyl phosphate and 0.4ml. of 0.4M acetate buffers of the following pH values: 3.75, 4.1, 4.5, 4.75, 5.0, 5.4, 5.75. Two series of "blanks" were also incubated. In the first of these the substrate, and in the second the enzyme, was replaced by an equal volume of water. The incubation period was thirty minutes. Phenol liberated was estimated by the method given earlier (p. 48).

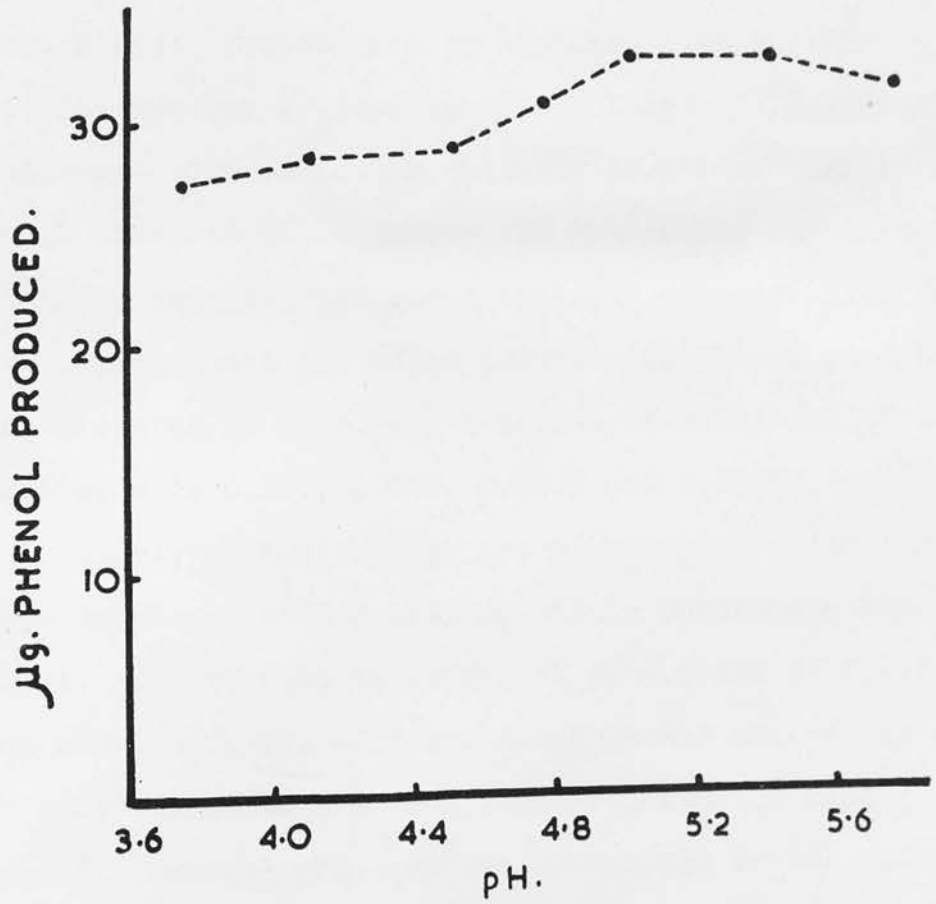
b) Results and Discussion: The experimental values obtained are graphed in diagram 11 . The curve appears to be very flat and the values for activities differ little over the range 4.5 to 5.4.

(ii) The Effect of Variation of Substrate Concentration.

The effect of variation of phenyl phosphate concentration on the amount of phenol liberated by a fixed amount of yolk sac membrane homogenate was determined. Two experiments were carried out, one with a preparation of activity approaching the maximum which could be determined accurately and one about the minimum. /

DIAG. II.

EFFECT OF pH. ON PHENOL PRODUCTION FROM PHENYL PHOSPHATE BY A YOLK-YOLK SAC HOMOGENATE IN ACETATE BUFFERS.



minimum. The experiments were carried out on preparations from different eggs.

At this point in the work, the use of a 1% sodium chloride solution 0.01N with respect to magnesium chloride as a medium for homogenisation and dilution of membrane was introduced to ensure comparable ionic strengths and magnesium ion concentrations as far as was possible in all experiments.

a) Method: Yolk sac membranes were excised from embryonated eggs, frozen hard in acetone-solid carbon dioxide and homogenised in five volumes of 1% sodium chloride-0.01M magnesium chloride solution. After a rough check of its activity the homogenate was diluted with the salt solution to a suitable concentration for the experiments. A molar solution of phenyl phosphate was prepared of pH 4.5. A series of dilutions of this was made to 0.20M, 0.04M, 0.008M and 0.0016M.

0.2ml. of the various phenyl phosphate solutions were incubated with 0.2ml. of dilute homogenate and 0.4ml. of 0.4M acetate buffer at pH 4.5 and 37° C for one hour. Series of substrate "blanks" and enzyme "blanks" were prepared and incubated for the same period. Phenol produced was determined by the standard procedure.

b) Results: The results of these experiments are given in /

in diagram 12. The molarity of phenyl phosphate shown is that of the solution as incubated. In diagrams 12(A) and (B) it should be noted that the substrate concentration is plotted on a logarithmic scale. The amount of phenol liberated by the more concentrated enzyme appears to attain an optimum value in the region 0.01M to 0.05M and to slowly decrease with increasing phenyl phosphate concentration above this range, indicating some degree of substrate inhibition. The decrease in activity as the highest substrate concentration is reached is much more marked with the dilute enzyme preparation.

It would appear from these experiments that in the region 0.01 to 0.05 molarity of phenyl phosphate, the variation in the amount of phenol liberated is minimal.

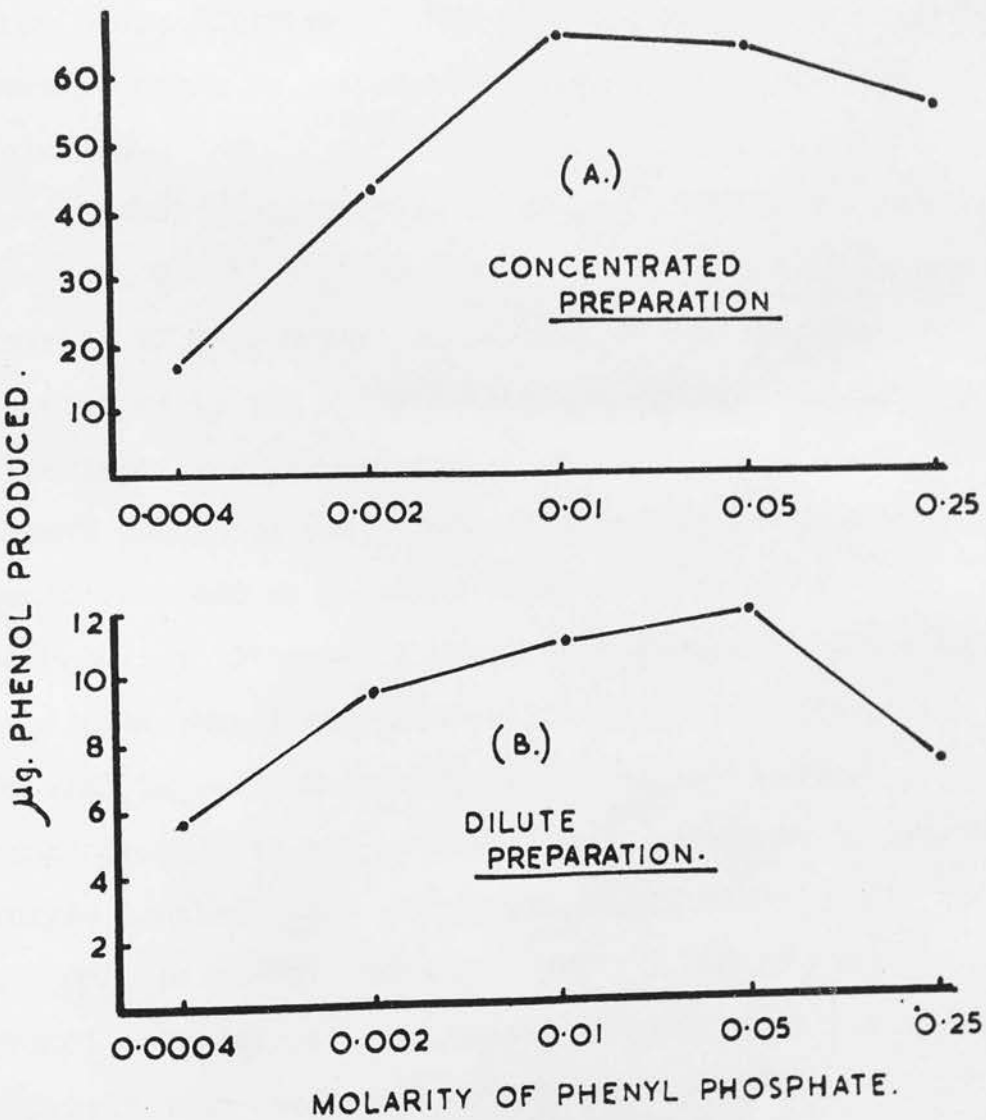
(iii) The Effect of Variation of Enzyme Concentration.

Experiments were now carried out to find a substrate concentration at which a linear relationship between enzyme concentration and phenol liberated existed. The previous experiment indicated that molarity from 0.01 to 0.05 would be most suitable.

a) Experimental Method: /

DIAG. 12.

EFFECT OF VARIATION OF PHENYL PHOSPHATE CONCENTRATION ON PHENOL LIBERATED BY YOLK-YOLK SAC ACID PHOSPHATASE.



a) Experimental Method: Homogenates were prepared as in the preceding experiment, and after a rough check on their activity were diluted with 1% sodium chloride-0.01M magnesium chloride to give approximately 40µg. phenol per hour when incubated under the standard conditions of pH 4.5 and 37°C with 0.01M substrate (p. 48). From these dilutions, 0.50, 0.33 and 0.25 concentrations were prepared in sodium chloride-magnesium chloride solution.

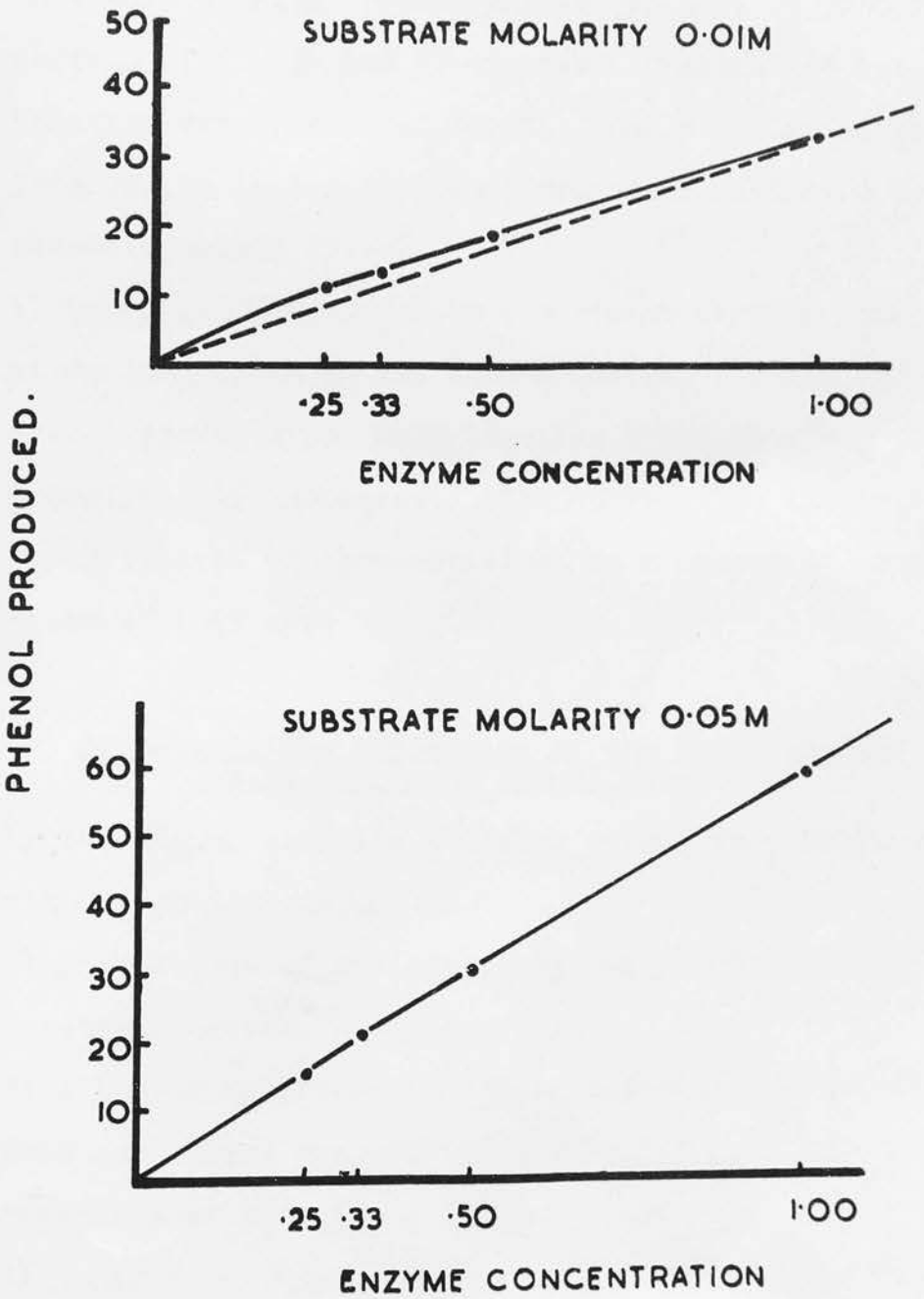
Phenol produced from 0.2ml. of 0.04M phenyl phosphate in 0.4ml. of 0.4M acetate buffer (pH 4.5) in one hour at 37°C by 0.2ml. of these preparations, was determined by the usual method (p. 48). A second experiment was carried out in which 0.2ml. of 0.2M phenyl phosphate was used as substrate, the experimental conditions and so on being otherwise identical.

b) Results: Diagram 13 gives values for phenol liberated from phenyl phosphate in these two experiments. It will be seen that using 0.05M phenyl phosphate concentration in the incubates, the relationship between enzyme concentration and phenol liberated is linear up to 60µg. of phenol produced. With 0.01M substrate, phenol produced per unit enzyme concnetration decreases slightly with increasing enzyme concentration.

(iv) The Effect of Variation of the Hydrolysis Period /

DIAG. 13.

EFFECT OF VARIATION OF ENZYME CONCENTRATION ON PHENYL PHOSPHATE HYDROLYSIS BY YOLK SAC MEMBRANE ACID PHOSPHATASE.



(iv) The Effect of Variation of the Hydrolysis Period on Phenol Produced.

a) Method: A membrane homogenate was prepared as in the preceding two groups of experiments and 0.2ml. portions incubated with 0.2ml. of 0.2M phenyl phosphate in 0.4ml. of 0.4M acetate buffer (pH 4.5) at 37°C for periods of 21, 39 and 57 minutes. Enzyme and substrate "blanks" were incubated for the same periods and phenol from enzyme action on phenyl phosphate estimated by the standard method (p. 48).

b) Results: Values for an experiment of this kind are given in diagram 14 and show a decided falling off in phenol produced per unit time, as the time given for hydrolysis is increased. The curve given in diagram 14 is typical of those obtained in a number of experiments of this type.

(v) Summary of the Properties of the Yolk Sac Acid Phosphatase Acting on Phenyl Phosphate.

1) The enzyme exhibits activity over a wide pH range with an optimum around pH 5.

2) Some degree of inhibition by the phenyl phosphate substrate occurs.

3) A linear relationship exists between phenol liberated and enzyme concentration with 0.05 molarity of substrate at pH 4.5.

4) Under /

4) Under the conditions given in 3) the amount of phenol produced per unit time falls markedly after 30 minutes.

4. THE ACID PHOSPHATASE ACTIVITY OF THE YOLK SAC MEMBRANE AT VARIOUS STAGES OF INCUBATION:

The experiments detailed in the previous section enabled conditions to be chosen, so that the activity of the acid phosphomonoesterase of the yolk sac membrane at different stages of incubation could be compared using phenyl phosphate as a substrate. The following standardised technique was adopted and applied to membranes from eggs of 7 to 19 days' incubation period.

(i) Method of Estimating Acid Phosphatase Activity.

The eggs were frozen hard in acetone-solid carbon dioxide and stripped of their shells. The yolk sac membranes were excised, with as little accompanying yolk as possible, weighed and homogenised in a known volume of ice-cold 1% sodium chloride-0.01M magnesium chloride solution. In the case of the 19 day eggs, the whole of the yolk sac was taken for homogenisation, the separation of the residual yolk from the membrane being extremely difficult with them. An aliquot of the prepared homogenate was then diluted with a measured volume of sodium chloride-magnesium chloride solution to give a level of activity suitable for estimation.

The /

The effect of freezing and treatment in the automix was checked in an experiment reported in Appendix I (p. 94).

The activity was found under the following conditions: 0.2ml. of the preparation in a centrifuge tube were treated with 0.4ml. of 0.4M acetate buffer at pH 4.5 and placed in a water bath at 37°C for five minutes before addition of 0.2ml. of 0.2M phenyl phosphate solution of pH 4.5. After 30 minutes at 37°C, 2ml. of 1:5 diluted Folin-Ciocalteu reagent were introduced, the contents mixed and removed from the bath. Protein precipitated was packed in the centrifuge and 2ml. of the supernatant fluid transferred to a tube containing 4ml. of 1N sodium carbonate. Details of the process of colour development and the determination of optical density and phenol value have been described on p. 48. Enzyme "blanks" in which 0.2ml. of water replaced the phenyl phosphate solution, and substrate "blanks" in which the enzyme was replaced by an equal volume of water, were incubated and analysed by the same procedure. The phenol value of these was subtracted from that obtained with enzyme and substrate together. All assays including the "blanks" were done in duplicate.

The activity of the yolk sac membrane was calculated and expressed as milligrams of phenol produced in

15 /

15 minutes at 37° C and pH 4.5 by its entire enzyme content in a solution 0.05 molar with respect to phenyl phosphate and 0.2 molar with respect to acetate buffer. (Unit of King and Armstrong 1934).

(ii) Results and Conclusions.

The activities of the yolk sac membranes of a number of eggs of incubation periods from 7 to 19 days are given in table XV with values for the activity per gramme wet weight of membrane, calculated using Byerly's values for the membrane weight (Byerly 1932).

It will be seen that the activity rises steadily as does the activity per unit weight of membrane throughout the period studied. The activity of the acid phosphatase of the yolk sac membrane appears to parallel the growth of the embryo.

5. STUDIES ON PARTIALLY PURIFIED ACID PHOSPHATASE FROM THE YOLK SAC MEMBRANE:

An attempt was now made to obtain a stable preparation of acid phosphatase from the yolk sac membrane, which was free from phosphorus compounds and proteolytic activity, and to test its specificity using a variety of compounds including lipovitellin and some of its degradation products.

(i) Purification of the Enzyme. /

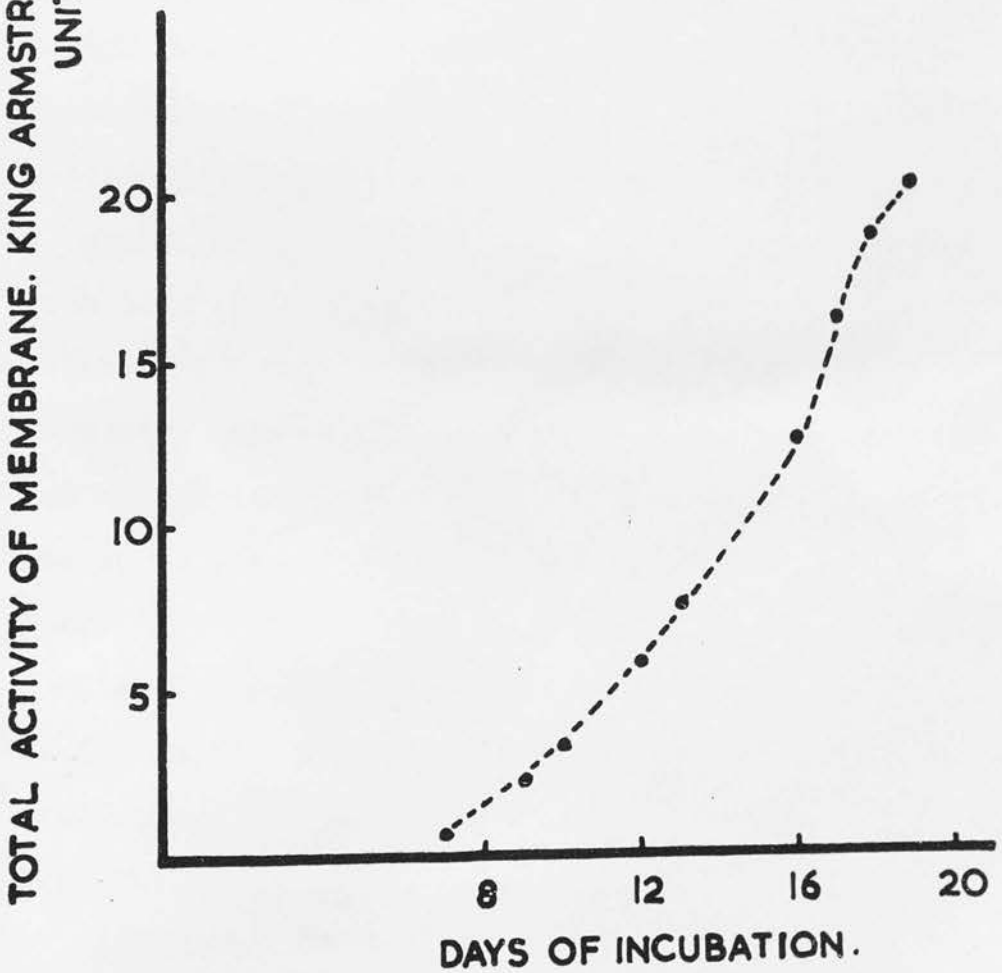
TABLE XV.

Acid Phosphatase Activity of the Yolk Sac Membrane at Various Stages of Incubation

Day of Incubation	Acid Phosphatase Activity King & Armstrong Units	Acid Phosphatase Activity per unit wet wt. of Yolk Sac Membrane	Acid Phosphatase Activity per unit wet wt. of Embryo
7	0.56 0.65 0.75	0.63	0.74
9	2.2 2.2	1.62	0.96
10	3.5 3.05	1.83	0.99
12	5.9	2.05	0.83
13	7.55	2.38	0.67
16	12.6	3.15	0.79
17	15.5 17.2	3.97	0.84
18	19.0	4.65	0.91
19	20 17.5 24.6	7.3	0.77

DIAG. 15.

ACID PHOSPHATASE ACTIVITY OF THE YOLK SAC MEMBRANE AT VARIOUS STAGES OF INCUBATION.



(i) Purification of the Enzyme.

Ammonium sulphate fractionation of membrane homogenates proved disappointing. The protein precipitates so obtained were difficult to centrifuge and carried down the phosphatase roughly in proportion to their bulk. The activity of the aqueous or saline extracts of acetone powders of the yolk sac membrane was very low. Dialysis followed by low temperature acetone fractionation proved the most suitable approach of the methods tried, and the following procedure was adopted.

a) Method of Enzyme Purification: Yolk sac membranes from 14 or 15 day eggs were homogenised with four volumes of water in the automix for five minutes. The resulting preparation was dialysed in cellophane bags against distilled water for 48 hours before centrifuging for 40 minutes at 3000 r.p.m. (temperature 0°C.). Three layers were obtained - a heavy protein precipitate, a slightly cloudy supernatant liquid and a yellow layer of fat on the surface of the liquid. The protein precipitate and the fat layer were rejected. The supernatant liquid, after treatment with an equal volume of M/20 potassium hydrogen phthalate was cooled to -10°C before the addition over a period of 30 minutes of 30ml. of acetone per 100ml. of solution (final volume on addition of phthalate), followed by 12 grammes of magnesium /

magnesium chloride hexahydrate per 100ml. After four hours at -5°C, the material was centrifuged at that temperature for 40 minutes at 3000 r.p.m. The precipitate was rejected. The supernatant, freed from acetone by suction at the pump (the rapid evaporation of the acetone keeping the material cold) was followed by dialysis for 16 hours in the refrigerator against frequent changes of distilled water. A precipitate formed on dialysis was removed by centrifugation and rejected. The preparation was now extracted twice with ethyl oleate to remove some remaining suspended material, presumably fat, and separated again by centrifugation. After passage through a Seitz filter to effect sterilization, the clear colourless solution was stored in the refrigerator until used.

Concentration of the enzyme was effected by precipitation with four volumes of acetone at -20°C and centrifugation at -10°C, followed by suspension of the precipitate in a small volume of water, the acetone being removed by suction at the pump and dialysis.

b) The pH Activity Relations of the Partially Purified Enzyme: The possibility existed that the yolk sac membrane contained a number of different enzymes capable /

capable of hydrolysing phenyl phosphate and it was necessary to compare the action of the material prepared above on phenyl phosphate with that of an unfractionated preparation from the yolk sac membrane. As it was impossible to characterise the crude preparations by a Michaelis constant, the effect of variation of pH was taken as a criterion of identity. The activity of the partly purified material was therefore determined over a series of pH values and compared with the values obtained with a crude membrane homogenate.

Experimental Method: The activity of the partly purified preparation was determined at a number of pH values from 3.75 to 5.75 in acetate buffer, by a procedure identical with that used in the experiments on the pH activity relations of membrane homogenates (p. 55).

Results and Discussion: Values obtained in this experiment are compared with values from an unfractionated homogenate in diagram 16; activities being expressed as percentages of the maximum value obtained in each case.

Within the accuracy of the experimental method, the results appear to indicate that the purified preparation is identical in this property with the phenyl phosphatase of the membrane or is closely representative of enzymes in the membrane which hydrolyse phenyl phosphate /

phosphate in the pH range 3.75 to 5.75.

(ii) The Action of the Partly Purified Preparation on a Number of Phosphate Esters.

At this stage a study of the action of the partly purified acid phosphatase on a number of physiologically occurring ester monophosphates was made in the hope that the specificity of the enzyme might throw some light on its function in the living membrane.

a) Experimental: 0.02M solutions of glucose-1-phosphate, sodium glycerophosphate, adenosine-5-phosphate, yeast cytidylic acid and phenyl phosphate were prepared.

One millilitre portions of these solutions were incubated in centrifuge tubes at 37°C for periods of three and six hours, with one millilitre of partly purified phosphatase preparation and one millilitre of 0.4M acetate buffer pH 4.5. Enzyme and substrate "blanks" in which one millilitre of water replaced the substrates and enzyme respectively, were also incubated for the same periods.

After the desired period of incubation, 2ml. of 15% trichloroacetic acid was added to the tubes, the contents mixed and left at 0°C for twenty minutes before centrifuging. 2ml. portions of the supernatant fluid were taken for the determination of inorganic phosphate.

b) Results: The results of this experiment, given in diag. /

diag. 17 show that the preparation is capable of hydrolysing a variety of phosphate esters at rates comparable with that for its action on phenyl phosphate.

(iii) The Action of a Partly Purified Phosphatase Preparation on Lipovitellin and Some Products of its Degradation.

Although it was not found possible to obtain an acid phosphatase preparation of concentration comparable with that in a crude yolk sac membrane homogenate, it was felt that it would be interesting to find out if such partly purified preparations had any activity on lipovitellin and some products obtained from it by proteolytic action, and to compare the activity on these with that on a simple phosphate ester.

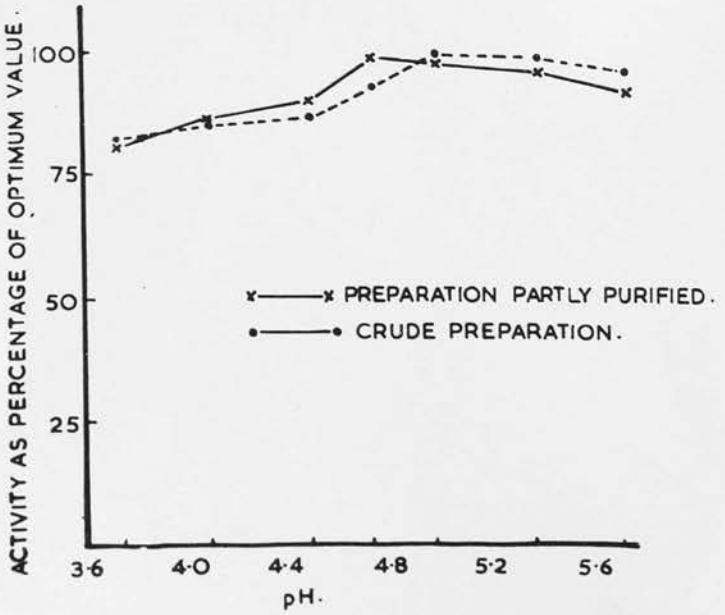
a) Experimental Method: Lipovitellin, haematogen and ovotyrin preparations containing 250µg. of phosphorus per ml. were made. Haematogen is the insoluble residue from pepsin treatment of vitellin. Ovotyrin is obtained by the action of trypsin on on the haematogen so produced. Methods of preparing these will be found in the appropriate Appendix (p. 96 et seq.).

A solution of sodium glycerophosphate 0.02M was prepared.

One millilitre of each of the suspensions or solutions was incubated with one millilitre of purified phosphatase preparation and one millilitre of 0.4M acetate /

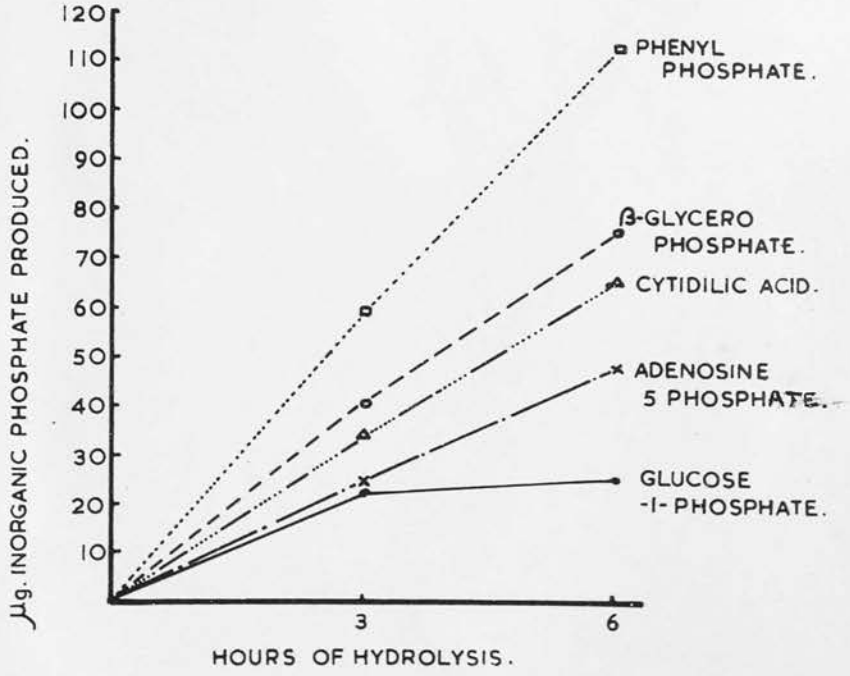
DIAG. 16.

COMPARISON OF pH. ACTIVITY RELATIONS OF CRUDE AND PARTLY PURIFIED ACID PHOSPHATASE OF THE YOLK SAC MEMBRANE.



DIAG. 17.

ACTION OF PARTLY PURIFIED YOLK SAC ACID PHOSPHATASE ON SOME PHOSPHATE ESTERS.



acetate buffer at pH 4.5. The experiment and analysis was carried out in an identical manner to that of the previous experiment with simple esters, the period of incubation, however, being extended to 16 hours.

b) Results: In the cases of lipovitellin, haematogen and ovotyrin, a slight turbidity in the final solution for analysis precluded the determination of the optical densities. The solutions were, however, completely devoid of the molybdenum blue colour indicating that no inorganic phosphate had been produced. From the glycerophosphate, 0.369mg. of inorganic phosphate (expressed as phosphorus) were liberated.

It may be concluded that, if the preparation has any action on lipovitellin or derived phosphopeptides, it is of a very much lower order than that on glycerophosphate and other simple phosphate esters.

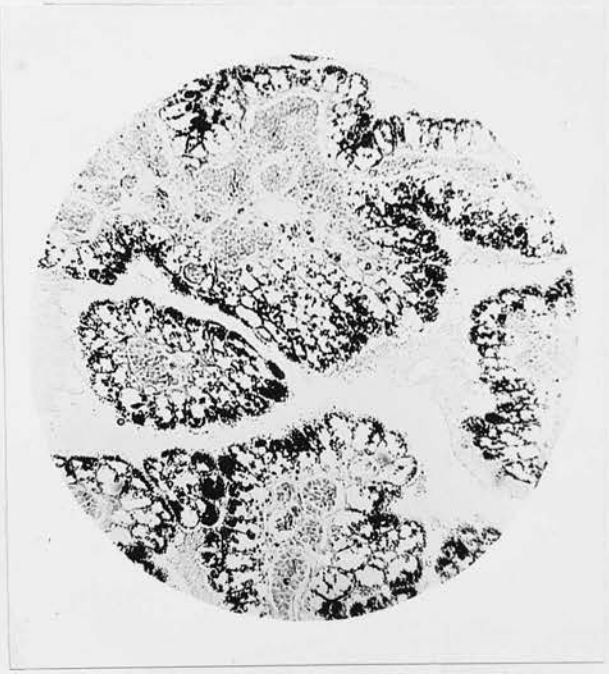
6. THE HISTOCHEMICAL LOCALISATION OF THE YOLK SAC PHOSPHATASES:

It was felt that a useful confirmation of the existence of phosphomonoesterases in the cells of the yolk sac membrane might be obtained by the application of histochemical techniques. Portions of yolk sac membrane with some adherent yolk were fixed and dehydrated in anhydrous acetone. The method of Gomori (1941) was applied to the localisation of acid phosphatase /

phosphatase and of Gomori (1951) to the tracing of alkaline phosphatase. Photomicrograph (2) stained for acid phosphatase and counterstained with eosin shows an intense reaction in the cells of the yolk sac membrane bordering on the yolk. Photomicrograph (3), stained by the method for alkaline phosphatase demonstrates the presence of this enzyme in the same cells.

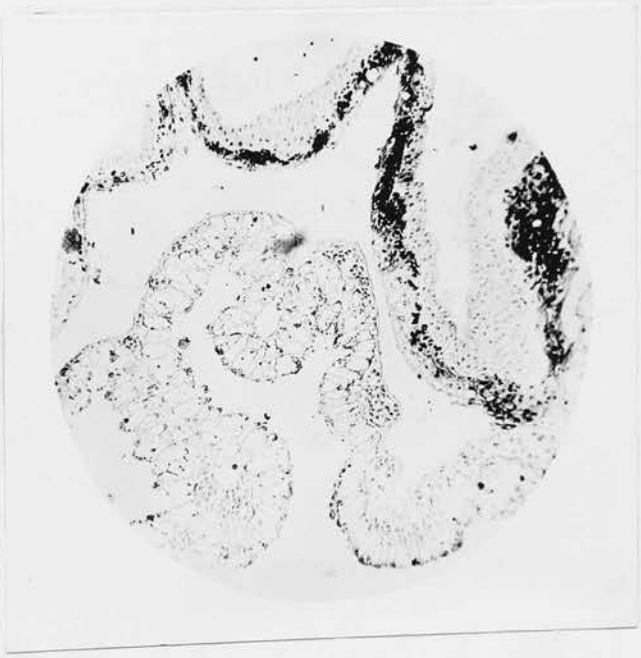
Photomicrograph (2)

Yolk Sac Membrane Stained For
Acid Phosphatase by Method of Gomori (1941)



Photomicrograph (3)

Yolk Sac Membrane Stained For
Alkaline Phosphatase by Method of Gomori (1951)



SUMMARY AND DISCUSSION

The work related in this section constitutes a preliminary study of the phosphomonoesterases of the yolk sac membrane. The method of King and Armstrong (1934) using sodium phenyl phosphate as substrate was adopted.

In experiments with crude yolk sac preparations, hydrolytic activity towards phenyl phosphate was shown over a wide range with optima about pH 4 to 5 and pH 10. Residual yolk preparations, however, showed no activity. The activity per unit weight of haem iron was determined for blood in the vascular network of the membrane and from this, the activity of a membrane homogenate due to blood from the small vessels was calculated and compared with the experimentally determined value for the homogenate. In this way, it was possible to show that membrane homogenates had much more acid and alkaline phosphatase activity than could be accounted for by the blood present, and therefore that the phosphatases existed in the cells of the yolk sac membrane itself. This was later confirmed by a histological technique.

Attention was now directed towards some of the general properties of the acid phosphomonoesterase of the membrane, and the dependence of the amount of hydrolysis /

hydrolysis on pH, phenyl phosphate concentration, enzyme concentration and time of incubation. Conditions were found under which the activity, as determined by the amount of phenol produced from phenyl phosphate, formed a measure of the enzyme concentration.

It was now possible to carry out a study of the acid phosphatase activity of the yolk sac membrane at various stages of incubation. The results of this investigation showed that the activity of the enzyme did not vary directly with the growth of the membrane but appeared to run roughly parallel to the wet weight of the embryo, the ratio of phosphatase activity to embryo wet weight showing a maximum deviation from its mean value of only 30% over a period in which the embryo grows from approximately 1g. to 30g. It seems plausible to assume that the enzyme is in some way closely connected with the growth requirements of the embryo.

It was hoped that some clue as to the metabolic function of the enzyme might be found in its specificity. A partially purified preparation of the enzyme was made and tested a number of phosphate esters: glucose-1--phosphate, adenosine-5-phosphate, yeast cytidylic acid and glycerophosphate; all of which appeared to be hydrolysed at rates comparable with the activity on phenyl phosphate. It would appear, therefore, that the preparation /

preparation consisted of an enzyme of wide specificity or a number of related enzymes.

Experiments on the effect of this partially purified preparation on lipovitellin and some products from the action of proteolytic enzymes on it, indicated that the enzyme had little or no activity on the phosphoprotein or derived products. It was not, however, found possible to obtain a partially purified preparation of phenyl phosphatase activity as high as a simple membrane homogenate and so the possibility that the enzyme may play a part in phosphoprotein breakdown is not rigidly excluded.

To sum up, the yolk sac membrane contains both acid and alkaline phosphomonoesterases, the former at least of wide specificity towards simple phosphate esters. The activity of the acid phosphatase roughly parallels the growth of the embryo. The function of the enzyme remains conjectural.

DISCUSSION

THE NUCLEIC ACIDS OF THE EMBRYO

It is not proposed to discuss the values for nucleic acid phosphorus in the embryo in great detail. The amount of information which may be extracted from values, which represent the sum of nucleic acids of a variety of different organs and types of tissue must necessarily be limited; but it may be pointed out that if the hypothesis of Vendrely and Vendrely (1948, 1949), that the deoxyribonucleic acid content of all somatic cells in an organism is constant, be accepted, the results obtained in Part I form a measure of embryonic growth in terms of cell number and show a considerable decrease in the rate of cell division in the embryo towards the end of incubation.

THE UTILISATION OF PHOSPHOPROTEIN

In Part I of this thesis, mean values of the phosphorus of phosphoproteins in the "Remainders" of eggs at various stages of incubation were obtained. Analysis of embryos and "Remainders" from which the yolk and yolk sac were removed, gave only very small quantities of material appearing as phosphoprotein phosphorus. The results obtained therefore represent phosphoprotein within the yolk sac and, at the same time, phosphoprotein in the whole system. The results show a very considerable disappearance of phosphoprotein phosphorus during development.

From the data for the phosphoprotein phosphorus level at the various stages of incubation, the rate of disappearance of phosphoprotein phosphorus was calculated. This rate is, of course, not necessarily the rate of utilisation of phosphorus from phosphoprotein by the embryo. Until the 10th day the embryo is smaller than the extra-embryonic structures and until the 17th day at least, a considerable quantity of the materials taken from both the white and the yolk must be incorporated into the membranes. The evidence of the experiments in Part II of this thesis indicates that the phosphorus of phosphoproteins is liberated extra-embryonically /

extra-embryonically by the yolk sac membrane. It seems probable that this membrane at least must derive some of the phosphorus for its nucleic acids, and possibly structural phospholipids, from the metabolism of phosphoprotein and that these materials are degraded and made available when the yolk sac membrane regresses, and help to meet the high demand for phosphorus in the embryo during calcification of the bones. The comparatively early peak of the rate of phosphoprotein breakdown, about 13 days, makes the idea of partial extra-embryonic utilisation and storage extremely plausible.

The investigations reported in Part II of this thesis were designed to learn something of the method and location of the metabolism of phosphoprotein. Analysis of blood draining the yolk sac failed to demonstrate the presence of phosphoprotein and although the method of analysis was perhaps not sufficiently sensitive to entirely eliminate the possibility of direct transport of phosphoprotein to the embryo for metabolism, attention was focussed on the possibility of extra-embryonic metabolism. Experiments in which yolk from fresh eggs was incubated either with or without homogenisation, failed to show any redistribution of phosphorus among the groups analysed, and would seem to indicate that the enzymes responsible for phosphoprotein breakdown are not located in the yolk. The

experiments /

experiments do not, however, eliminate the possibility of a small highly localised enzyme system existing in the periphery of the yolk and capable of supplying the small embryonic requirements for phosphorus at the earliest stages of incubation, but they do indicate that qualitatively important changes in the amount of phosphoprotein are not caused by enzymes contained in the yolk. The avian egg in this respect differs from that of the frog (Harris 1946) in which a phosphoprotein phosphatase is associated with the yolk particles and becomes active on homogenisation.

Experiments performed with early residual yolk confirm that the yolk does not contain enzymes attacking phosphoprotein. It should be pointed out, however, that the method used would not detect the conversion of native phosphoprotein to acid-insoluble phosphopolypeptides. The investigations of Remotti (1927) indicate that proteolytic activity until the last few days of incubation is not a function of the yolk.

When late residual yolks were incubated, it was found that some slight breakdown of phosphoprotein took place. It is of interest that Remotti (1927) has shown extracellular proteolysis in the yolk during the last few days of incubation.

As the greater part of phosphoprotein breakdown occurs /

occurs before activity can be demonstrated in the yolk itself, metabolism must be occurring elsewhere. Examination of yolk sac membrane homogenates with yolk showed these to have considerable ability to break down phosphoprotein with the liberation of inorganic phosphate. It would seem, therefore, with the probable absence of phosphoprotein from the blood coming from the yolk and the non-activity of the yolk itself, that the metabolism of phosphoprotein is closely associated with the yolk sac membrane.

It remains now to say something of the enzymes responsible for the breakdown of phosphoprotein. From the nature of the substance these must be proteolytic enzymes and a phosphatase or phosphatases. Two possibilities would appear to exist:

1) Proteolysis precedes the removal of phosphorus with the phosphatases acting on phosphopeptides or serine phosphate.

2) The phosphorus of phosphoprotein may be removed before proteolysis and the protein residue is thereafter attacked by proteolytic enzymes.

In the work which has been done, the methods of analysis do not distinguish between native phosphoprotein and derived acid-insoluble polypeptides and therefore the results cannot give an answer as to whether some degree /

degree of proteolysis is a necessary prerequisite of the liberation of inorganic phosphorus or not. Such evidence as has been gathered can bear, therefore, only on the question whether proteolysis proceeds to a degree such as to produce acid-soluble phosphopeptides before the liberation of inorganic phosphate. It is, perhaps, unnecessary to sound the additional cautionary note that the breakdown of phosphoprotein observed in homogenates may not be identical to that in vivo.

If we assume the first possibility outlined above to be correct, then we might expect that inhibition of the second stage of the process, the phosphatase action, would lead to the accumulation of soluble phosphopeptides. Experiments in which fluoride or molybdate, both known inhibitors of phosphatase action, were added to yolk sac membrane-yolk homogenates led to inhibition of the phosphoprotein breakdown in its primary stage. This might, however, be due to inhibition of proteolytic action by the fluoride or molybdate. Experiments in which proteolytic action was followed by estimation of acid-soluble material reacting with Folin-Ciocalteu reagent, indicated that proteolysis was not inhibited by fluoride. It would thus appear that the liberation of inorganic phosphate occurs from the phosphoprotein or an acid-insoluble phosphopolypeptide. These experiments /

experiments have not been reported in full because serious objections exist. The validity of the phosphorus estimations in presence of the high fluoride concentrations used is doubtful and it is questionable whether the material estimated by Folin-Ciocalteu reagent in such a gross mixture of materials as the yolk sac homogenates is really tyrosine, and whether the tyrosine can be taken as an index of proteolysis of phosphoprotein. It is probable that the yolk sac membrane has a variety of proteolytic enzymes and quite possible that one necessary for the breakdown of phosphoprotein to a stage where the phosphorus can be liberated, may be inhibited while the production of tyrosine or other Folin-Ciocalteu reacting substance goes uninterrupted. It is the author's view that the sequence of enzyme action in phosphoprotein breakdown is still undecided and that such information as has been obtained is merely suggestive that the liberation of phosphorus takes place from the phosphoprotein or acid-insoluble polypeptides, and not from acid-soluble or low molecular weight phosphopeptide intermediates.

THE PHOSPHATASES OF THE YOLK SAC MEMBRANE

This part of the work has been discussed previously in the appropriate section and it is not proposed to comment further on the results obtained.

SUMMARY

A modification of the method of analysis of Schmidt and Thannhauser for the estimation of nucleic acid and phosphoprotein phosphorus has been developed and applied to the analysis of fresh hen eggs and embryos and "Remainders" of eggs, from seven days of incubation to hatching. The results of these analyses are discussed.

From results obtained in the analysis of blood from the large vessels of the yolk sac circulation, and an investigation of the activity of yolk, residual yolk and yolk sac membrane homogenates towards phosphoproteins, a hypothesis as to the method of utilisation of phosphoprotein in the embryonated hen egg is advanced. The existence of phosphomonoesterases of both the acid and alkaline type in the yolk sac membrane has been shown by chemical and histological methods, and the activity of the former determined in eggs from seven to nineteen days of incubation. A partial purification of the acid phosphatase has been effected and a measure of its specificity found.

The implications of the experimental work performed are discussed.

APPENDIX I

CHECKS ON THE ANALYTICAL PROCEDURES USED

THE VALIDITY OF THE ANALYTICAL TECHNIQUE USED IN DETERMINING NUCLEIC ACID AND PHOSPHOPROTEIN PHOSPHORUS

It is perhaps necessary at this point to recapitulate on the principle of this method. The method may be conveniently divided into three stages.

1) Removal of acid-soluble and lipoid phosphorus from a tissue homogenate.

2) Treatment of the residue from this with 1N sodium hydroxide at 37° C for 16 hours, when deoxyribonucleic acid remains unhydrolysed, ribonucleic acid is split to nucleotides and the phosphorus of phosphoprotein is converted to inorganic phosphate.

3) Estimation of total phosphorus on the incubate from 2) (giving the sum D.N.A.P. + R.N.A.P. + P.P.) and of total phosphorus (giving R.N.A.P. + P.P.) and inorganic phosphate (P.P.) on a centrifugate of the incubate after acidification.

A number of experiments were carried out testing the soundness of the technique adopted.

1) The reproduceability of the method determining inorganic phosphate was found. The method for total phosphorus had already proved reliable in this laboratory and was not further investigated.

2) Hydrolysis experiments were performed with purified nucleic acids and phosphoproteins to ensure that /

that the alkali treatment did leave deoxyribonucleic acid quantitatively acid-precipitable, hydrolysed ribonucleic acid completely to acid-soluble material with no liberation of inorganic phosphate, and converted the phosphorus of phosphoproteins fully to inorganic phosphate.

3) To ensure that the removal of acid-soluble and lipoid phosphorus in the procedure adopted was complete, or at least sufficiently exhaustive as to leave quantitatively insignificant amounts of these compounds, the amounts of phosphorus in the various trichloroacetic acid and lipid solvent extracts of embryo and "remainder" homogenates were determined and the effect of prolonged further extraction with these solvents investigated.

4) The possibility existed that the materials analysed might contain compounds other than the nucleic acids and phosphoproteins which were not removeable by the solvents employed. As a partial check on this point, the liberation of acid-soluble and inorganic phosphate from a solvent extracted embryo homogenate with 1N sodium hydroxide at 37° C was followed. The hydrolytic pattern obtained was then compared with that of ribonucleic acid and the phosphoproteins.

5) Finally, recovery experiments were performed in which known amounts of nucleic acid and casein were added to homogenates and analysed.

1. THE /

1. THE DETERMINATION OF INORGANIC PHOSPHATE:

For the determination of phosphoprotein phosphorus in the embryo, it was necessary to have a method for estimating inorganic phosphate which would give quantitative results on amounts of phosphorus of the order 5µg. Preliminary experiments with a simple technique, involving precipitation of the inorganic phosphate as the calcium salt at pH 9, followed by washing the precipitate with dilute ammonium hydroxide solution, having given inadequate recoveries at the 10µg. level, the method described on p.13 was evolved.

This method involves co-precipitation with calcium carbonate from a solution containing a fairly high calcium ion concentration at pH 10. This is followed by washing the precipitate with 1N ammonium hydroxide containing calcium chloride. It was felt that the high pH and large calcium ion concentration in both the precipitating medium and the washing solution would decrease the solubility of the calcium phosphate, while the bulky precipitate from the co-precipitation would minimise mechanical loss.

Recovery Experiment:

A solution of potassium dihydrogen phosphate was prepared containing 10µg. phosphorus per ml. 0.5ml. and 1.0ml. volumes of this solution were pipetted into centrifuge /

centrifuge tubes containing 1ml. of 0.3N hydrochloric acid (equivalent to the amount of acid in the aliquots taken for routine analysis). Inorganic phosphate was then determined as on p. 13.

Ten experiments were carried out at each level (5µg. and 10µg.), the recoveries being 101.1% S.D. 2.4% at 5ug. and 100.4% S.D. 2.9% at 10µg.

2. TREATMENT OF NUCLEIC ACIDS AND PHOSPHOPROTEINS AT 37° C WITH NORMAL SODIUM HYDROXIDE:

These experiments were carried out to test the validity of the alkaline hydrolytic separation used in the analytical method.

(i) Treatment of Deoxyribonucleic Acid at 37°C with Normal Sodium Hydroxide.

a) Method: Approximately 20mg. of ox thymus deoxyribonucleic acid were weighed, 10ml. of 1N sodium hydroxide added and the resulting solution incubated at 37°C. One ml. aliquots were removed at intervals during the incubation and, after chilling, treated with 1ml. of trichloroacetic acid-hydrochloric acid mixture (p. 11). After thirty minutes the precipitated material was packed by centrifugation and a 1ml. portion of the supernatant taken for analysis for total acid-soluble phosphorus. Total nucleic acid phosphorus was determined on 1ml. of a 1:6 dilution of the sodium hydroxide solution.

b) Results: /

b) Results: Results for experiments of this type, given in table A.1., show that the amounts of deoxyribonucleic acid degraded in 16 hours with sodium hydroxide at 37°C (the conditions used in the analytical procedure) are negligible.

(ii) Hydrolysis of Ribonucleic Acid at 37°C with Normal Sodium Hydroxide.

a) Method: About 30mg. of yeast ribonucleic acid were weighed and treated with 50ml. of 1N sodium hydroxide at 37°C. Aliquots of the solution were removed at intervals and after chilling, treated with an equal volume of trichloroacetic acid-hydrochloric acid mixture. Two ml. portions of the centrifugate from this were taken for the determination of inorganic phosphate and 1ml. portions for the estimation of acid-soluble phosphorus.

b) Results: Table A.2. gives the results of an experiment of this type. The hydrolysis of this type of nucleic acid to acid-soluble material is complete in much less than 16 hours. The small amount of inorganic phosphate produced (less than 2%) does not constitute a serious error in the determination of ribonucleic acid, but would form quite an important contribution to phosphoprotein phosphorus in analyses of embryo homogenates.

(iii) Hydrolysis /

TABLE A.1.

Treatment of Thymus Deoxyribonucleic Acid with 1N Sodium Hydroxide at 37°C.

Period of Treatment		Percentage Hydrolysed to Acid-soluble Phosphorus	
Hours	Mins.	First Experiment	Second Experiment
	10	0.56	0.70
56		0.71	0.91
108		1.96	1.68
144		3.82	3.90

TABLE A.2.

Treatment of Yeast Ribonucleic Acid with 1N Sodium Hydroxide at 37°C.

Period of Treatment		Percentage Hydrolysed to	
Hours	Mins.	Acid-soluble Phosphate	Inorganic Phosphate
	5	75	0.0
	10	97	0.0
2		100	0.0
20		-	1.7
72		-	3.4

(iii) Hydrolysis of Casein and Vitellin at 37°C by Normal Sodium Hydroxide.

a) Method: Approximately 300mg. of the phosphoprotein was weighed and treated with 50ml. of 1N sodium hydroxide at 37°C. Two ml. aliquots were removed at intervals during incubation, chilled, treated with an equal volume of trichloroacetic acid-hydrochloric acid mixture and centrifuged. A 1ml. sample of the centrifugate was analysed for inorganic phosphate. A total phosphorus determination was done on the incubate when all the protein had dissolved: 0.5ml. portions being taken for analysis.

b) Results: In table A.3. results of experiments of this type done with casein and vitellin are given. It will be seen that the hydrolysis to inorganic phosphate is complete in less than 16 hours.

3. EXPERIMENTS TESTING THE EFFICIENCY OF THE EXTRACTION PROCEDURES USED IN REMOVING ACID-SOLUBLE AND LIPOID PHOSPHORUS:

a) Method: Samples of embryo and "remainder" homogenates were taken through the extraction procedure given on p. 12. Total phosphorus was estimated on aliquots or on the whole of each extract. In the case of lipid solvent extracts, the solvent was removed by evaporation in a current of air on a water bath. Residual chloroform-methanol extractable material was determined on a number /

TABLE A.3.

Treatment of Vitellin and Casein with 1N Sodium Hydroxide at 37°C.

Period of Treatment		Percentage Hydrolysed to Inorganic Phosphate	
Hours	Mins.	Vitellin	Casein
	5	11	-
	10	-	31
	15	29	-
	30	-	65
1	10	-	87
1	50	84.5	-
6	30	100.1	100.25

number of residues from the standard procedure by grinding lightly with a glass rod before refluxing with chloroform-methanol for a period of two hours. Residual acid-soluble materials were obtained by treating the dried residue from the standard procedure with ice cold 7.5% trichloroacetic acid for two hours.

b) Results: Table A.4. gives typical results of experiments of this type. The order of acid-soluble or lipoid phosphorus remaining after the standard extraction procedure is 1-3% of the phosphorus of the nucleic acids in embryo homogenates, or of the phosphoprotein phosphorus of "remainder" homogenates. It was felt that this error could not be eliminated without considerably lengthening the method, and perhaps introducing a new source of error in degradation and loss of the materials to be estimated.

4. HYDROLYSIS EXPERIMENTS ON THE EXTRACTED RESIDUE FROM EMBRYO HOMOGENATES:

It was felt that a study of the rate of liberation of acid-soluble and inorganic phosphate from residues from an embryo homogenate might give evidence as to whether the nucleic acids of the embryo behaved similarly to those of ox thymus and yeast and afford a method of detecting the presence of compounds other than the nucleic acids and phosphoproteins in the residues.

a) Method: /

TABLE A.4.

Phosphorus in Extracts of
Embryo and "Remainder" Homogenates

	Embryo	"Remainder"
Nature of Extract	Total Phosphorus in Extract µg.	Total Phosphorus in Extract µg.
Trichloroacetic Acid	603	80.0
Trichloroacetic Acid	29.0	21.5
Trichloroacetic Acid	2.3	7.2
Trichloroacetic Acid	1.4	2.8
Ethanol	227	-
Alcohol-ether	-	870
Alcohol-ether	8.0	60.9
Alcohol-ether	1.7	5.3
Chloroform-methanol	1.7	6.6
Chloroform-methanol	1.2	3.1
Residual Acid-soluble	0.8	1.6
Residual Chloroform--methanol soluble	0.7	2.8

a) Method: Dry extracted residues from equal quantities of 10, 15 and 20 day embryos were pooled. The mixture was incubated at 37° C with 1N sodium hydroxide. 2ml. aliquots were withdrawn at intervals and treated with 2ml. of trichloroacetic acid-hydrochloric acid. After 30 minutes at 0° C, the precipitate formed was centrifuged down and 1ml. aliquots of the supernatant taken for the determination of acid-soluble and inorganic phosphorus. When the residue was completely in solution 0.5ml. were taken for the determination of total phosphorus.

b) Results: Table A.5. gives results of an experiment of this type. It will be seen that the organic acid--soluble phosphorus is nearly constant after one hour and inorganic phosphate reaches constancy after four hours. Comparison of the results with those obtained using purified ribonucleic acid and phosphoprotein shows that they are at least compatible with the substances undergoing hydrolysis being ribonucleic acid and phosphoprotein.

5. RECOVERY EXPERIMENTS:

As a final check on the validity of the method, it was necessary to perform some full recovery experiments in which portions of homogenate were analysed with and without the addition of known amounts of nucleic acids and /

TABLE A.5.

Treatment of Extracted Residues
from Embryo Homogenates
with 1N Sodium Hydroxide at 37°C.

Period of Treatment Hours	Mins.	Acid-soluble Phosphorus Produced µg.P.	Inorganic Phosphate Produced µg.P.
	5	7.0	0.5
	10	13.3	0.7
	30	18.2	1.4
1	20	19.3	1.9
4	0	19.5	2.4
20	0	19.7	2.4
Total Phosphorus 34.9µg.			

and phosphoprotein.

a) Method: One ml. portions of an embryo homogenate were pipetted into a series of tubes. To some of these were added measured volumes of solutions containing known amounts of D.N.A., R.N.A. and casein phosphorus. The contents of the tubes were analysed by the standard procedure.

b) Results and Discussion: Table A.6. gives results of experiments of this type. Recoveries of casein and deoxyribonucleic acid phosphorus were reasonably satisfactory at 95%. Recoveries of over 80% were never obtained with ribonucleic acid, but it is probable that the commerical samples of yeast nucleic acid used in the experiment had suffered extensive degradation in their preparation and consequently were much more acid-soluble than the native material, resulting in losses during the trichloroacetic acid extractions. The work of Calvery (1928) indicates that the ribonucleic acid of the chick embryo is present as ribonucleoprotein. The efficacy of trichloroacetic acid as a protein precipitant renders it unlikely that there would be appreciable losses of such complexes. The finding that the extracted residue from an embryo homogenate containing some hundred microgrammes of ribonucleic acid phosphorus gave, on further extraction with trichloroacetic acid for /

TABLE A.6.

Recovery Experiments with Deoxyribonucleic Acid, Ribonucleic Acid and Casein on Analytical Method for Nucleic Acid and Phosphoprotein

	Deoxy-Ribonucleic Acid Phosphorus µg.	Ribonucleic Acid Phosphorus µg.	Phospho-protein Phosphorus µg.
Homogenate	58 60	114 110	14 15
Homogenate + 88µg. D.N.A.P.	145 140	112 112	13 16
Mean Recovery of Deoxyribonucleic Acid Phosphorus = 142.5-59 = 83.5µg. = 95%			
Homogenate	55 61	107 106	15 14
Homogenate + 176µg. R.N.A.P.	57 58	238 238	15 16
Mean Recovery of Ribonucleic Acid Phosphorus = 238-106.5 = 131.5µg. = 74.5%			
Homogenate	58 60	114 110	14 15
Homogenate + 73.6µg. Casein P.	57 62	113 114	84 86
Mean Recovery of Casein Phosphorus = 85-14.5 = 70.5µg. = 95.8%			

for two hours under the conditions used in the method, only about 1µg. of acid-soluble material, seems to eliminate the possibility of ribonucleic acid being degraded and lost during the acid extraction. Loss during the lipid solvent extractions seems very unlikely.

6. SUMMARY OF RESULTS OF EXPERIMENTS ON THE VALIDITY OF THE METHOD OF ANALYSIS FOR NUCLEIC ACIDS AND PHOSPHOPROTEIN:

1) A mean recovery of 100.4% S.D. 2.9% was obtained on standard phosphate solutions using the method for inorganic phosphate described on p. 13.

2) Deoxyribonucleic acid gave less than 1% of acid--soluble material when incubated with normal sodium hydroxide at 37° C for 56 hours.

3) Ribonucleic acid was rendered completely acid--soluble under the action of normal sodium hydroxide at 37° C in less than one hour and gave less than 2% of inorganic phosphate in 20 hours.

4) Casein and vitellin phosphorus was completely converted to inorganic phosphate in six hours with normal sodium hydroxide at 37° C.

5) Residues after the extraction procedure gave analytically negligible quantities of acid-soluble and lipoid phosphorus on prolonged further extraction.

6) In hydrolysis experiments with the extracted residue /

residue from embryo homogenates, the organic acid-soluble phosphorus reached a constant level after one hour and the inorganic phosphate after four hours under treatment with normal sodium hydroxide at 37° C, confirming in some measure that the substances undergoing hydrolysis were ribonucleic acid and phosphoprotein.

7) Recoveries of 95% were obtained in experiments in which known amounts of casein and deoxyribonucleic acid were added to embryo homogenates and analysed. In similar experiments with yeast ribonucleic acid, recoveries were less than 80%. Reasons are advanced for believing that losses of ribonucleic acid are considerably less in the estimations on embryo material.

THE EFFECT OF FREEZING AND HOMOGENISATION ON THE ACTIVITY OF YOLK SAC ACID PHOSPHATASE

It was found convenient in determining acid phosphatase activity in the yolk sac membrane to freeze the eggs and excise the material for homogenisation. It was necessary to check that freezing, thawing and homogenisation did not result in partial destruction of the enzyme.

a) Method: An embryonated egg was frozen hard and, after dilution with sodium chloride-magnesium chloride solution, homogenised in the automix for one minute. A portion (1) was withdrawn, the remainder of the homogenate was returned to the automix and treated for a further three minutes after which a second portion (2) was withdrawn. The homogenate remaining was frozen hard in acetone-carbon dioxide and thawed twice before a further five minutes treatment in the homogeniser (3).

The activities of (1), (2) and (3) were then determined by the usual method (p. 48).

b) Results and Discussion:

Phenol liberated by preparation (1) = 35.7µg.

Phenol liberated by preparation (2) = 35.5µg.

Phenol liberated by preparation (3) = 35.3µg.

There is an apparent slight loss of activity on freezing and homogenisation, but the small difference between (1) frozen once and homogenised for 1 minute and /

and (3) frozen three times and homogenised for 9 minutes is scarcely significant.

APPENDIX II

PREPARATION OF MATERIALS

a) Lipovitellin and Vitellin:

These were prepared essentially by the method of Calvery and White (1931-32). Lipovitellin was stored as a suspension in water for periods of not more than three days before use. Traces of ether in this preparation were carefully removed by repeated evacuation.

b) Casein:

B.D.H. "Light White Soluble" Casein was dissolved in sodium bicarbonate solution or ammonia and precipitated by the addition of hydrochloric acid. This process was repeated, the reprecipitation being facilitated by the addition of some ethanol. The casein was then washed with ethanol and ethanol-ether. After air drying and grinding in a mortar, the protein was refluxed with chloroform-methanol mixture, 1:1 by volume, and collected on a Buchner funnel for washing with ethanol-ether, 2:3 by volume, and ether. The air dried product was then stored over calcium chloride.

c) Ox Thymus Deoxyribonucleic Acid:

The sample used was gifted to the author by Dr. C.M. Mauritzen. It had been prepared by the method of Mirsky and Pollister (1942) and purified by treatment with ribonuclease.

d) /

d) Yeast Ribonucleic Acid:

This was a sample of yeast ribonucleic acid (L. Light and Co.) which was further purified by the method of Kunitz (1940).

e) Cytidylic Acid:

This was a sample prepared from yeast nucleic acid by Dr. A.B. Roy and given to the author.

f) Glucose-1-Phosphate:

This was prepared by the Honours Biochemistry Class 1951-52, under the direction of Dr. A.B. Roy, by the potato phosphorylase method and gave theoretical figures for phosphorus.

g) Adenosine-5-Phosphate:

This was material marketed by the Roche Chemical Company and was not further treated.

h) Haematogen (cf. Bunge 1885):

6g. of vitellin were added to 60ml. of 0.1N hydrochloric acid containing 2g. of pepsin scales and incubated for 4 days at 37° C. The insoluble residue from this treatment was centrifuged down, washed three times with 0.1N hydrochloric acid, then twice with alcohol-ether 2:3 and finally with ether, before drying.

Analysis /

Analysis by Allen's method (Allen 1940) gave 7.5% phosphorus.

i) Ovotyrin:

0.4g. of haematogen were treated with 10ml. of sodium bicarbonate solution and 0.5g. of commercial trypsin at 37°C for 72 hours. After acidification with glacial acetic acid, the product was filtered and treated with lead acetate solution. The resulting precipitate was centrifuged down and decomposed with sodium carbonate solution. After filtering, the solution was saturated with sodium chloride and acidified with hydrochloric acid. The precipitated material was washed with alcohol and then ether, before drying. The percentage of phosphorus in this product was 6.1.

APPENDIX III

THE SOURCE AND QUALITY OF REAGENTS USED

All reagents other than those mentioned below were of analytical reagent quality and were used without further purification.

a) A.R. Reagents Which Were Further Treated:

Acetone was refluxed with potassium permanganate, distilled, dried with potassium carbonate and redistilled.

Ethanol was refluxed over sodium hydroxide pellets for several hours before redistilling.

Chloroform was washed several times with water in a separatory funnel, dried over sodium sulphate and distilled.

b) Reagents Not Of A.R. Quality But Used Without Further Treatment:

Amidol	Photographic developer (Johnsons Ltd.)
Ethyl oleate	
Folin-Ciocalteu Reagent	Prepared solution (B.D.H. Ltd.)
Sodium glycerophosphate	(B.D.H. Ltd.)
Sodium phenyl phosphate	(B.D.H. Ltd.)
Sodium metabisulphite	Griffin and Tatlock.
Tetra-azotised-o- Dianisidine	A sample kindly gifted by I.C.I. Ltd. (Dyestuffs Division).

c) /

c) Reagent Solutions Prepared At Frequent Intervals:

Amidol reagent	Three days.
Folin-Ciocalteu reagent dilution 1:5	Daily.
Trichloroacetic acid 15%	Two days.

APPENDIX IV

TABLES OF ANALYTICAL RESULTS

TABLE I.

Results of Nucleic Acid Analyses of Embryos

Embryo Age	Embryo Weight	D.N.A. as Phosphorus mg.	R.N.A. as Phosphorus mg.	D.N.A. Calculated mg.	R.N.A. Calculated mg.
6½	0.66	0.056	0.136	0.60	1.53
7½	1.18	0.175	0.260	1.89	2.92
8	1.30	0.140	0.259	1.51	2.90
8	1.64	0.204	0.355	2.20	3.99
8½	1.86	0.222	0.413	2.40	4.65
9½	2.43	0.294	0.540	3.17	6.08
9½	2.56	0.342	0.584	3.69	6.58
9½	2.51	0.347	0.628	3.75	7.05
10½	3.82	0.513	0.930	5.55	10.4
11	4.75	0.630	1.20	6.81	13.5
11½	5.08	0.886	1.35	9.58	15.2
11½	5.05	0.715	1.32	7.72	14.8
11½	5.39	0.834	1.60	9.02	18.0
11½	5.15	0.810	1.44	8.75	16.2
11½	5.59	0.862	1.84	9.32	20.7
13	8.28	1.33	2.96	14.7	33.2
13½	10.35	1.45	2.83	15.7	31.8
13½	10.34	1.58	2.64	17.1	29.6
14	11.3	1.60	3.00	17.3	33.6
14	11.6	1.79	2.72	19.3	30.6
15½	16.7	2.44	4.62	26.4	51.0
15½	16.4	2.51	4.49	27.1	50.2
15½	16.1	2.51	4.05	27.1	45.5
16	17.6	2.68	4.21	29.0	47.5
16½	19.5	2.66	4.74	28.8	53.4
17½	22.6	3.30	5.00	35.6	56.2
17½	21.8	2.94	4.88	31.7	55.0
17½	21.9	3.09	4.98	33.4	56.0
18½	26.3	3.89	4.98	42.0	56.0
18½	27.0	3.09	4.82	33.4	54.3
19	29.4	3.11	4.99	33.6	56.1
19	27.5	3.35	5.08	36.1	57.0
19	30.5	3.38	5.80	36.5	65.1
19	28.8	3.11	5.62	33.6	63.2

TABLE II.

Results of Phosphoprotein Analyses of "Remainders"

Embryo Weight g.	Period of Incubation	mg. Phosphoprotein Phosphorus in "Remainder"	Mean Value
Unincubated Eggs	0	30.3	
	0	25.8	
	0	32.5	
	0	28.9	
	0	31.3	
	0	27.3	
	0	29.4	
	0	27.6	
	0	26.6	
	0	28.0	27.8
	0	32.0	
	0	29.3	
	0	23.5	
	0	23.4	
	0	26.1	
	0	27.5	
	0	26.5	
	0	29.2	
0.5	6	24.5	24.5
0.5		24.5	
1.0	7	30.5	25.7
1.2		21.5	
1.0		26.8	
1.2		27.4	
1.3		22.1	
1.4	8	27.3	26.2
1.3		26.7	
1.2		22.5	
1.0		27.6	
1.6		26.9	
2.0	9	28.6	26.1
2.0		28.2	
1.9		29.8	
2.4		27.8	
2.5		20.1	
2.5		22.2	
2.9 /			

Embryo Weight g.	Period of Incubation	mg. Phosphoprotein Phosphorus in "Remainder"	Mean Value
2.9 2.9 2.9 2.5 3.7 3.1 3.3 3.1 3.0 3.0 3.0 4.0 3.8	10	27.4 26.0 30.5 27.5 24.6 24.2 23.7 22.6 24.9 25.6 32.2 25.4 24.5	26.1
4.0 3.9 4.0 4.8 4.1 5.1 5.0 5.4 5.6	11	26.0 25.0 25.8 17.6 29.0 24.5 22.6 22.0 26.5	24.3
6.4	12	24.8	
9.4 9.1 8.3 8.2 10.2 10.5 10.6 10.3 10.4	13	19.9 19.4 22.7 21.5 17.5 14.2 11.8 22.4 14.6	18.2
11.3 11.6 12.3 12.4 13.0 13.0	14	10.5 16.2 20.2 14.0 17.0 17.6	15.8
13.6	15	13.0	
17.7 /			

Embryo Weight g.	Period of Incubation	mg. Phosphoprotein Phosphorus in "Remainder"	Mean Value
17.7 16.7 16.4 17.7 18.9 19.5 18.9 18.8 19.3	16	9.1 6.9 8.7 14.2 11.9 11.8 16.2 14.8 12.8	11.8
19.5 20.0 20.4 21.1 19.8 22.5 22.0 21.9 20.0 22.0 22.6 22.0 21.8 21.9	17	6.8 10.4 11.3 4.5 11.5 5.4 11.6 10.6 8.9 12.6 4.8 6.3 9.2 11.0	9.6
24.7 23.1 27.0 27.0 26.3 26.0 23.8 26.8	18	12.7 5.6 6.0 8.1 6.6 6.6 6.8 11.7	8.0
28.2 30.0 30.0 28.6 27.4 30.9 30.0	19	4.6 4.5 6.6 7.2 8.5 4.8 6.2	6.1
34.4 35 35 35	20- 21	4.2 5.0 2.1 9.9	5.3

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